Renal Dysfunction and the Pathogenesis of Cardiovascular Disease

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Renal Dysfunction and the Pathogenesis of Cardiovascular Disease

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Frank Stam

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	dr. K. de Meer

“When you treat a disease, you can win or loose.
When you treat a patient, I guarantee you, you’ll win, whatever the outcome…”

Dr. Patch Adams, Gesundheit! Institute, West Virginia, United States of America

Aan Eleonoor,
Deborah en Ymke

CONTENTS

Chapter 1

General introduction

INTRODUCTION

Chronic kidney disease is a world-wide health problem. In the Netherlands, in 2005, approximately 13,000 people had end-stage renal disease of whom 45% were treated with chronic renal replacement therapy (either with haemodialysis or peritoneal dialysis) and 55% had received a renal transplant [1]. The number of people with end-stage renal disease is rising with 4-5% annually [1]. Earlier stages of chronic kidney disease are even more prevalent.

Patients with end-stage renal disease have a high mortality, mainly due to cardiovascular disease, which is 10 to 30 times higher compared to the general population [2]. More recently, it has been shown that earlier stages of chronic kidney disease are also associated with an increased total and cardiovascular mortality [3–5]. The causes of cardiovascular disease are diverse in chronic kidney disease, including many well known (traditional) cardiovascular risk factors like older age, male sex, hypertension, higher low-density lipoprotein cholesterol and lower high-density lipoprotein cholesterol plasma levels, diabetes mellitus, smoking, physical inactivity, family history of cardiovascular disease and left ventricular hypertrophy [6]. Most of these traditional risk factors have a high prevalence in chronic kidney disease [7]. However, risk equations based on people without chronic kidney disease have been shown to underestimate the actual cardiovascular risk in patients with chronic kidney disease [8–10]. Therefore, it is conceivable that other, non-traditional cardiovascular risk factors, like hyperhomocysteinaemia, endothelial dysfunction, low-grade inflammation and higher blood levels of advanced glycation end-products, are involved in the pathogenesis of cardiovascular disease in chronic kidney disease. In this thesis, studies are presented which aimed to elucidate the relationship between renal function and these non-traditional cardiovascular risk factors.

Homocysteine is an amino acid which may lead to cardiovascular disease through various proposed mechanisms, which include increasing oxidative stress, smooth muscle cell proliferation, coagulation and platelet activation, and decreasing endothelial function, gluthathione peroxidase activity and the vasodilator response to nitric oxide. Homocysteinaemia is a continuous independent risk factor for ischaemic heart disease and stroke in the general population [11]. Many determinants of the plasma homocysteine concentration have been identified. Nutritional deficiency of B-vitamins (e.g. folic acid, pyridoxine, cobalamin), genetic polymorphisms (e.g. $C_{677}T$ transition of 5,10-methylenetetrahydrofolate reductase, 844ins68 variant of cystathionine β-synthase, $A_{2756}G$

transition of methionine synthase), drugs (e.g. anticonvulsants, methotrexate, penicillamine), certain diseases (e.g. hypothyroidism), older age and male sex have been associated with hyperhomocysteinaemia, while malnutrition and hyperthyroidism have been associated with a lower plasma homocysteine concentration [12]. Plasma homocysteine concentration is also and strongly related to glomerular filtration rate [13]. Hyperhomocysteinaemia occurs in 85 to 100% of patients with end-stage renal disease [14–16]. The cause of hyperhomocysteinaemia in chronic kidney disease, especially the role of the kidney, is not completely understood. In ***Chapter 2***, homocysteine metabolism (Figure) in chronic kidney disease is discussed. In

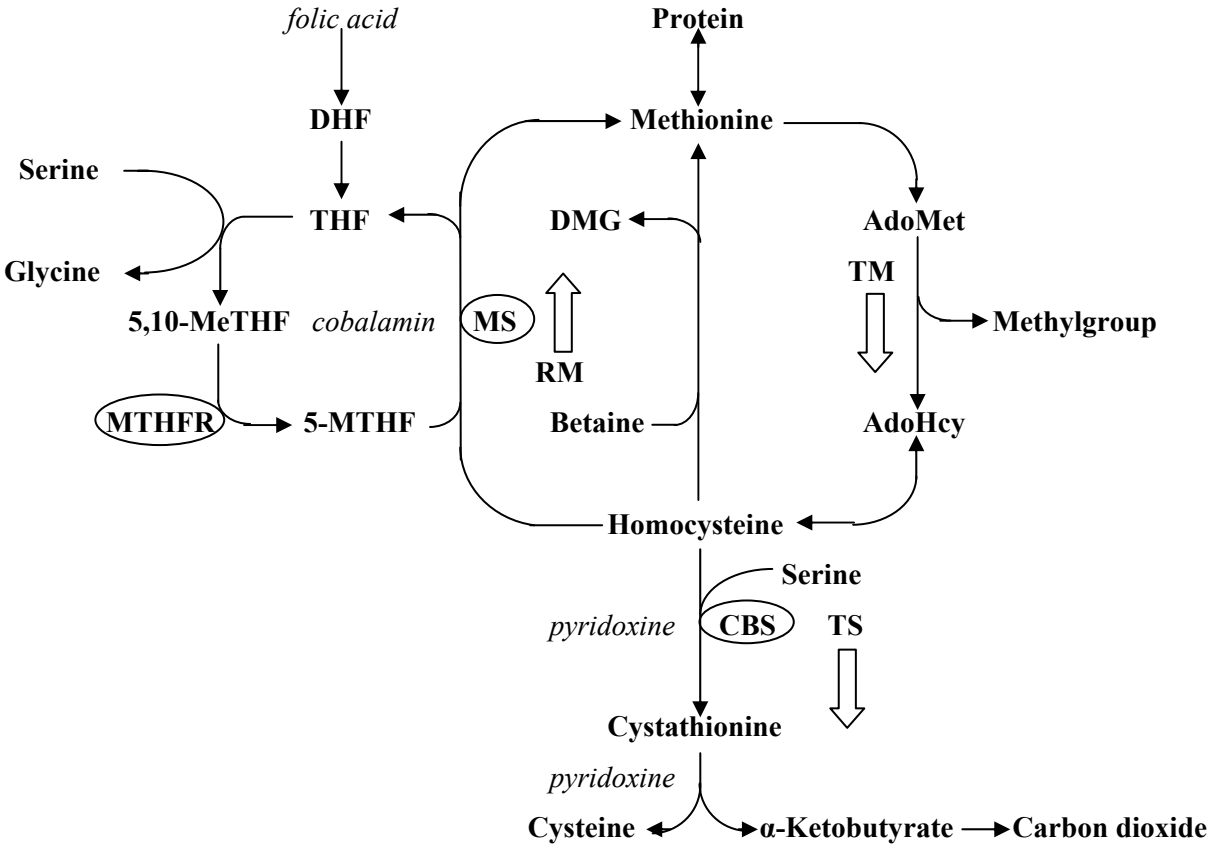

Figure. An overview of homocysteine metabolism. A concise overview of homocysteine metabolism, with open arrows indicating metabolic fluxes, ovals indicating enzymes and italics indicating vitamins. Homocysteine is the transmethylation (TM) product of the essential sulphur-containing amino acid methionine, with S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) as intermediates. Homocysteine can be either remethylated to methionine or degraded by transsulphuration (TS). In the folate-dependent remethylation reaction, which is catalyzed by methionine synthase (MS) and uses cobalamin as a cofactor, 5-methyltetrahydrofolate (5-MTHF) donates a methyl group to homocysteine. Subsequently, tetrahydrofolate (THF) receives a methylene group from the serine/glycine couple, a reaction that uses pyridoxine as a co-factor. Tetrahydrofolate can also be generated by reduction of supplemented synthetic folic acid to dihydrofolate (DHF) and subsequently to THF. Next, 5,10-methylenetetrahydrofolate (5,10-MeTHF) is reduced to 5-MTHF, requiring the enzyme methylenetetrahydrofolate reductase (MTHFR). Another folate-independent remethylation reaction, which is quantitatively unimportant in humans in physiological conditions [17], uses betaine as a methyl group donor, generating dimethylglycine (DMG). In the irreversible, catabolic (transsulphuration) pathway, the rate-limiting reaction is catalyzed by cystathionine β-synthase (CBS) and requires the active form of pyridoxine as a cofactor.

Chapter 3, whole body homocysteine metabolism is studied in end-stage renal disease patients, using a stable isotope labelled methionine tracer infusion technique, to test the hypothesis that one-carbon fluxes (methionine transmethylation, homocysteine remethylation and homocysteine transsulphuration) and metabolic homocysteine clearance are decreased in end-stage renal disease patients compared to healthy individuals. In addition, we explored the relationship of one-carbon flux rates with blood levels of the homocysteine precursors S-adenosylmethionine and S-adenosylhomocysteine. In ***Chapter 4*** and ***Chapter 5***, the effect of folic acid treatment on homocysteine metabolism is described in end-stage renal disease and healthy individuals, respectively, to investigate whether one-carbon flux rates and metabolic homocysteine clearance and blood levels of homocysteine can be increased. In healthy individuals the effect of folic acid on S-adenosylmethionine and S-adenosylhomocysteine was also studied.

In atherogenesis, endothelial dysfunction and (low-grade) inflammation are important and interrelated early steps [18]. There is evidence that a decreased renal function is associated with endothelial dysfunction and inflammatory activity [19,20]. In ***Chapter 6***, the relation between impaired renal function and plasma concentrations of biochemical markers of endothelial dysfunction and/or of inflammatory activity is described in a cross-sectional study of non-diabetic patients with a wide range in renal function.

Evidence accumulates that atherosclerosis may be an inflammatory disease, in which immune mechanisms interact with metabolic risk factors [21]. A high blood concentration of advanced glycation end-products, a heterogeneous group of compounds derived from the non-enzymatic reaction between glucose or other reducing sugars and proteins, has been suggested to be one of these metabolic factors [22,23]. Advanced glycation end-products may be involved in atherogenesis by acting with specific receptors for advanced glycation end-products [24] and accumulation in the vascular matrix [22]. There are limited data indicating that plasma levels of advanced glycation end-products are increased in chronic kidney disease, especially in end-stage renal disease [25,26]. In ***Chapter 7***, the relations between on the one hand the plasma level of advanced glycation end-product-peptides (incomplete metabolites of advanced glycation end-products) and on the other hand renal function and biochemical markers of endothelial dysfunction and inflammatory activity are investigated in the same population as in Chapter 6.

Several biochemical markers of endothelial dysfunction and inflammatory activity have been shown to be independent risk factors for cardiovascular morbidity and mortality in patients without chronic kidney disease [27–31]. It is unknown, whether the elevated

cardiovascular risk in chronic kidney disease is (partially) attributable to endothelial dysfunction and inflammation. In ***Chapter 8,*** we investigated whether endothelial dysfunction and inflammatory activity are related to mildly impaired renal function in a population-based study. Furthermore, in a prospective follow-up of the same population, it was investigated whether endothelial dysfunction and inflammatory activity contributed to cardiovascular and all-cause mortality associated with a lower glomerular filtration rate.

In ***Chapter 9***, these findings are critically discussed and placed in perspective.

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Chapter 2

Homocysteine metabolism in renal failure

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ABSTRACT

Of the many amino acid abnormalities that are present in chronic renal failure, hyperhomocysteinemia has drawn increasing attention because of its proposed role in the development and/or progression of atherothrombotic disease. Renal function is a major determinant of fasting plasma homocysteine level, and the inverse relationship between the glomerular filtration rate and plasma homocysteine level is present throughout the whole range of renal function. Although this suggests an active renal homocysteine metabolism, no important urinary excretion or active homocysteine extraction has been demonstrated in the human kidney. Analysis of plasma concentrations of the various cofactors and substrates of homocysteine metabolism, and the effects of different therapies indicate that an abnormal folate metabolism may be the cause of hyperhomocysteinemia in uremia. This is further supported by the finding that homocysteine remethylation, as assessed by stable isotope techniques, is impaired in dialysis patients. It is unclear whether decreased remethylation is also responsible for other abnormalities of homocysteine metabolism in renal failure such as the exaggerated rise and the impaired decline of plasma homocysteine concentration after methionine or homocysteine loading. More studies are necessary to pinpoint the precise mechanisms that lead to hyperhomocysteinemia in renal failure. This should lead to optimal treatment and, ultimately, to the prevention of cardiovascular complications in this vulnerable patient group.

Chronic renal failure has consistently been associated with abnormalities in plasma and intracellular amino acid concentrations. Some of these changes are likely to be caused, at least partially, by a defective synthesis, degradation or clearance by the kidneys themselves. For example, normal kidneys produce serine and arginine [1] and low levels of these amino acids are usually found in subjects with renal failure [2]. Additional factors that may influence amino acid levels are related to malnutrition, or to the uremic state per se, in which uremic toxins could affect enzyme activities, impair membrane transport or alter protein-binding.

Methionine is an essential sulfur amino acid derived from food or from the breakdown of endogenous protein. The biochemical pathway in which it is converted into homocysteine is called transmethylation. In this pathway, methionine first forms S-adenosylmethionine (AdoMet), which donates its activated methyl group to a variety of acceptors to form S-adenosylhomocysteine (AdoHcy). AdoHcy is subsequently hydrolyzed to homocysteine. In humans, methionine is the only precursor for homocysteine. Homocysteine can either be remethylated to methionine or be transsulfurated to cysteine. In remethylation, homocysteine receives a methyl group from 5-methyltetrahydrofolate or from betaine. Cobalamin is a necessary cofactor in the folate-dependent remethylation. The two reactions in transsulfuration, from homocysteine to cystathionine and from cystathionine to cysteine, require pyridoxal phosphate as the cofactor. Cysteine can be incorporated into protein and used for the synthesis of compounds such as taurine and glutathione, or be metabolized and excreted as sulfate. Key regulators of this metabolism are AdoMet, which activates homocysteine transsulfuration and inhibits its remethylation, and AdoHcy, which inhibits methionine transmethylation.

Renal function is an important determinant of the plasma homocysteine concentration. Various methods to estimate glomerular filtration rate (GFR), such as serum creatinine, creatinine clearance, plasma iohexol clearance, ^{51}Cr-EDTA clearance or plasma cystatin C, have unequivocally shown that declining renal function is associated with higher plasma homocysteine levels. This relationship is strongest when methods of assessing GFR that are more precise than serum creatinine are used. It has also been shown that the increase in plasma homocysteine with age is likely to be caused, at least partly, by the age-related decline in renal function [3]. The inverse relationship between plasma homocysteine and GFR seems to be independent of the primary renal disease and is present throughout the whole range of renal function, extending from normal to end-stage renal failure (ESRD), but remarkably also from normal to the hyperfiltering state in diabetic subjects [4]. Patients with end-stage renal disease usually exhibit plasma total homocysteine levels that are at least three times the upper

normal value, which is 12 to 15 μmo1/L in most laboratories. As in healthy subjects, about 75% of homocysteine in plasma in uremic patients is protein-bound. The free fraction consists of homocysteine-cysteine mixed disulfide, homocystine (that is, the oxidized dimer of homocysteine) and, to a minor degree, of reduced homocysteine. The plasma concentration of the latter does not seem to be increased in renal failure [5].

Hyperhomocysteinemia is not the only abnormal finding in renal patients when plasma concentrations of the various substances involved in methionine-homocysteine metabolism are considered. In contrast to the hypermethioninemia found in classical homocystinuria, caused by cystathionine β-synthase deficiency, methionine levels in renal failure are usually reported to be normal, whereas the metabolites of transsulfuration, that is, cystathionine and cysteine, are invariably elevated. Plasma concentrations of the intermediates of transmethylation, AdoMet and AdoHcy, are increased in hemodialysis patients, but AdoHcy more so than AdoMet, which results in a lower AdoMet to AdoHcy ratio compared to that in control subjects [6]. A similar pattern is found in erythrocytes: homocysteine and AdoHcy concentrations are increased and the AdoMet to AdoHcy ratio is reduced. In dialysis patients, the high intracellular AdoHcy levels are probably the result rather than the cause of the hyperhomocysteinemia [7]. Serum folate, vitamin B_{12} and vitamin B_6 1evels are usually normal in renal patients, whereas serine, an amino acid required in both transsulfuration and the folate cycle, is low. Serum levels of betaine and its demethylated product dimethylglycine seem to be normal in subjects with renal failure [8].

Several observations support the view that high plasma homocysteine levels in general are more likely the consequence than the cause of renal dysfunction. First, renal insufficiency is not a clinical feature of patients with classical homocystinuria, and these patients have very high plasma homocysteine levels of sometimes more than 200 μmol/L [9]. Second, higher plasma homocysteine levels are not associated with a more rapid decline of renal function [10].

Basically, two options are available to explain hyperhomocysteinemia in chronic renal failure. The first is a defective clearance or metabolism by the kidneys themselves, and the second is a systemic impairment in whole body homocysteine metabolism. These possibilities do not necessarily exclude each other. As urinary homocysteine excretion in humans is only minimal, that is, 3 to 10 μmo1/day [11] or 0.3% of creatinine clearance [12], loss of this excretion does not explain the hyperhomocysteinemia found in renal failure. In addition, the decline in the filtered homocysteine load in renal insufficiency is offset by an increase in fractional clearance [13], a common observation with amino acids. The hypothesis that

hyperhomocysteinemia in renal failure is caused by the loss of an active homocysteine catabolism in tubular cells is attractive in view of the large quantities of homocysteine that the tubules can derive from the glomerular ultrafiltrate [about 540 μmol/day, that is, 3 μmol/L (free homocysteine concentration) X 180 L/day (normal GFR)] or from the peritubular capillaries as well [about 2700 μmol/day, that is, 3 μmol/L x 900 L/day (normal ERPF)]. The presence of such renal metabolism can be studied in vivo by the renal arteriovenous difference technique. In postabsorptive rats, the total homocysteine concentration in the renal vein has been shown to be substantially (~20%) lower compared to that in the aorta [14]. In the absence of a sizeable urinary excretion, this indirectly proves that renal homocysteine catabolism does exist. The magnitude of the arteriovenous difference suggests uptake and conversion of the filtered homocysteine fraction only. In contrast to these findings, we did not find a significant renal arteriovenous difference in either free or total plasma homocysteine concentration in fasting humans with a normal renal function [1]. This discrepancy between the two species might relate to differences in the plasma free homocysteine fraction and the homocysteine metabolizing capacity of renal tissue. In rats, 75% of plasma homocysteine is in the free form and thus filterable, whereas in humans this fraction is only 25%. Furthermore, rat kidneys exhibit higher enzyme activities of cystathionine β-synthase and cystathioninase compared to human kidneys [15], which probably explains why homocysteine can readily be degraded to cysteine in the rat kidney [16]. Whether homocysteine metabolism occurs in the human kidney in non-fasting or uremic conditions remains to be investigated, but so far there is no evidence that loss of tubular homocysteine disposal is the cause of hyperhomocysteinemia in humans with renal failure.

Is hyperhomocysteinemia in renal failure then caused by a defective extrarenal or systemic homocysteine metabolism? Contributing factors in this scenario may be deficiencies of vitamins and/or substrates, genetic defects, and an altered total body homocysteine turnover. The role of vitamins and substrates can be studied by measuring their plasma (or intracellular) levels and by investigating the plasma homocysteine response after supplementation. Plasma levels of folate, cobalamin and pyridoxine status are generally adequate in renal failure patients, but apparently are insufficient to prevent hyperhomocysteinemia. Interestingly, the relation between these vitamin levels and plasma homocysteine is maintained in ESRD patients, but at a higher homocysteine level compared with subjects with normal renal function [17]. Of the three vitamins, folate is the strongest determinant of plasma homocysteine level in renal patients. This is also reflected by the results of several treatment regimens that have been investigated, which show that only folic-acid-containing therapy is

able to lower plasma homocysteine concentration in renal failure patients. Cobalamin is effective only when vitamin B_{12} levels are low, whereas vitamin B_6 has no significant effect on fasting plasma homocysteine. Serine deficiency would be a plausible candidate to explain hyperhomocysteinemia as plasma levels of serine, which is necessary both in transsulfuration and in the folate cycle, are low in renal failure. The results of serine supplementation in a small study, however, were disappointing [18]. A betaine-dependent remethylation defect also seems unlikely because plasma betaine levels are normal in renal failure and treatment with betaine alone or added to folic acid is not effective [19].

An important genetic determinant of plasma homocysteine level in the general population is the C677T mutation in the gene encoding for 5,10-methylenetetrahydrofolate reductase, which in its homozygous form is associated with higher homocysteine levels, especially in low folate states. In ESRD, the prevalence of the homozygous (TT) state is similar to that in the general population, about 10%. ESRD patients with the TT-variant have higher plasma homocysteine levels than wild-type or heterozygous patients, but in the latter groups, plasma homocysteine is still much higher compared to subjects with normal renal function. Until now, no specific genetic defects have been linked to hyperhomocysteinemia in renal disease. The third possibility, an altered whole body homocysteine handling, has been studied by oral methionine and homocysteine loading and by stable isotope techniques. Oral methionine loading, which is thought to stress the transsulfuration pathway, has been performed in subjects with chronic renal failure, renal transplant recipients and hemodialysis patients [13,20,21]. All studies have shown an exaggerated increase in plasma homocysteine level after the methionine load, which suggests a transsulfuration defect. In the hemodialysis patients, however, the postmethionine loading plasma homocysteine was significantly related to serum folate and not to vitamin B_6. In addition, folate therapy lowered both fasting and postmethionine-loading plasma homocysteine concentration, implying not only that folate-dependent remethylation is impaired in renal failure, but also that the remethylation and transsulfuration pathways are linked. After oral homocysteine loading, renal failure subjects exhibit a three- to fourfold increase in plasma homocysteine half-life compared to healthy controls [22]. This decreased plasma clearance did not significantly improve after folic acid therapy, although plasma homocysteine level was lower after treatment. Similar observations were made in dialysis patients after methionine loading [21]. The nature of this folate-resistant reduction in plasma homocysteine clearance in renal failure remains to be elucidated, but may relate to an impaired cellular uptake or intracellular catabolism of an acute homocysteine load. To quantitatively assess whole body transmethylation, remethylation and

transsulfuration rates in humans, stable isotope techniques can be applied. We have performed primed, continuous infusions of [$^{2}H_3$-methyl-l-^{13}C]methionine in fasting hemodialysis patients and healthy control subjects [23]. This tracer methionine has a $^{2}H_3$-methyl and a ^{13}C-carboxyl label, which are removed during transmethylation and oxidation (via transsulfuration), respectively. From the enrichments of the different isotopomers at steady state, the fluxes through the various pathways can be calculated. We found that remethylation and transmethylation were proportionally decreased in the dialysis patients, whereas transsulfuration was non-significantly decreased [23]. The latter showed an association with the vitamin B_6 status. The results strongly point to a defective total body homocysteine remethylation as the cause of hyperhomocysteinemia in renal failure. The pathogenesis of this impairment is not known, but may relate to direct inhibitory effects of uremic toxins or to an altered folate metabolism in uremia [24]. The influence of vitamin treatment on remethylation (and other) rates is currently under investigation.

In conclusion, several lines of evidence suggest that hyperhomocysteinemia in renal failure is due to impaired folate-dependent remethylation. At the same time, it should be stressed that this view may be too simplistic, as the regulation of the different pathways in methionine-homocysteine metabolism is not yet fully understood and probably is much more sophisticated and interrelated than presently assumed. Further studies on whole body and organ-specific abnormalities in homocysteine and folate metabolism and their regulatory factors are necessary to gain further insight in the pathogenesis of hyperhomocysteinemia in renal failure. This should lead to an optimal treatment and, hopefully, ultimately to a reduction of the occurrence of atherothrombotic disease in this high risk population.

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Chapter 3

Homocysteine clearance and methylation flux rates in health and end-stage renal disease: Association with S-adenosylhomocysteine

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ABSTRACT

Hyperhomocysteinemia is a risk factor for cardiovascular disease and occurs frequently in end-stage renal disease (ESRD), but its pathogenesis is poorly understood. We aimed to evaluate one-carbon flux rates of methionine and homocysteine (Hcy) in ESRD patients and healthy controls. Transmethylation (TM), remethylation (RM) and transsulfuration (TS), as well as Hcy clearance by TS (i.e., TS/plasma total Hcy concentration) and by RM (i.e., RM/plasma total Hcy concentration) were evaluated in relation to body composition, vitamins, and S-adenosylhomocysteine (AdoHcy) and S-adenosylmethionine (AdoMet) levels. After a fixed protein diet for three days, primed-continuous infusion of [$^{2}H_{3}$-methyl-1-^{13}C]methionine was performed in the postabsorptive state in 12 hemodialysis patients and 16 healthy volunteers. Hcy clearance by TS (–80% , $P < 0.001$) and by RM (–77% , $P < 0.001$) in ESRD patients were decreased as compared with healthy controls. The absolute flux rates of TM (–27%, $P < 0.01$) and RM (–28%, $P = 0.02$) were lower in the ESRD patients. After adjustment for age, TS was not significantly reduced. Whole blood AdoHcy was significantly elevated in ESRD and was a significant determinant of TM (standardized $\beta = –1.24$, $P = 0.01$), and RM (standardized $\beta = –1.43$, $P = 0.03$). In conclusion, patients with ESRD have impaired Hcy clearance by TS and RM. Elevated whole blood AdoHcy levels are associated with impaired RM and TM flux rates in these patients, and AdoHcy may be a key regulatory compound in one-carbon flux.

INTRODUCTION

Hyperhomocysteinemia is an independent cardiovascular risk factor in patients with end-stage renal disease (ESRD), with a prevalence reported as high as 85–100% [1]. Treatment regimens containing folic acid decrease plasma homocysteine (Hcy) concentration in ESRD patients [2–4], but only a small proportion of treated patients attain circulating Hcy levels in the normal range. The persistence of hyperhomocysteinemia in ESRD patients with supraphysiological folate levels is poorly understood. Elucidation of the regulation of the methionine-Hcy metabolism is necessary to understand the pathophysiology of hyperhomocysteinemia in patients with chronic renal failure and to develop more effective therapies.

In the one-carbon cycle (Figure 1), Hcy is the transmethylation (TM) product of the essential sulfur-containing amino acid methionine, with S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) as intermediates. Hcy can be either remethylated to methionine or degraded by transsulfuration (TS). There are two remethylation (RM) reactions in which Hcy is involved. In one reaction, methionine synthase converts Hcy into methionine with 5-methyltetrahydrofolate as methyl donor and reduced vitamin B_{12} as a cofactor; 5-methyltetrahydrofolate is generated by a reaction catalyzed by the enzyme 5,10-methylenetetrahydrofolate reductase. In the other RM reaction, betaine is used as methyl donor. In the irreversible catabolic (TS) pathway, the rate-limiting reaction is catalyzed by cystathionine β-synthase, requiring the active form of vitamin B_6 as a cofactor.

Patients with chronic renal failure have a substantially decreased plasma disappearance rate of Hcy after Hcy loading [5]. Diminished renal function (i.e., glomerular filtration rate) is strongly correlated with elevated plasma Hcy levels over a wide range of glomerular filtration capacity [6], suggesting a central role for the kidney in Hcy metabolism. As urinary Hcy excretion is negligible [7,8], loss of renal TS capacity has been hypothesized as a cause of hyperhomocysteinemia in renal failure [9,10]. However, no net renal extraction of Hcy occurs [11], which does not exclude Hcy metabolism in the kidney with a zero balance. Alternatively, whole body Hcy metabolism may be impaired in ESRD. In a previous pilot study, we have reported that the in vivo flux rates of RM and TM were diminished in patients with ESRD compared to healthy controls on the same protein intake, whereas TS was not significantly different [12]. The latter finding could have been due to the small number of individuals studied. It was argued by Hoffer [13] that the TS rate is not an adequate index of the whole body Hcy disposal capacity, i.e., the metabolic Hcy clearance by TS. Therefore, in

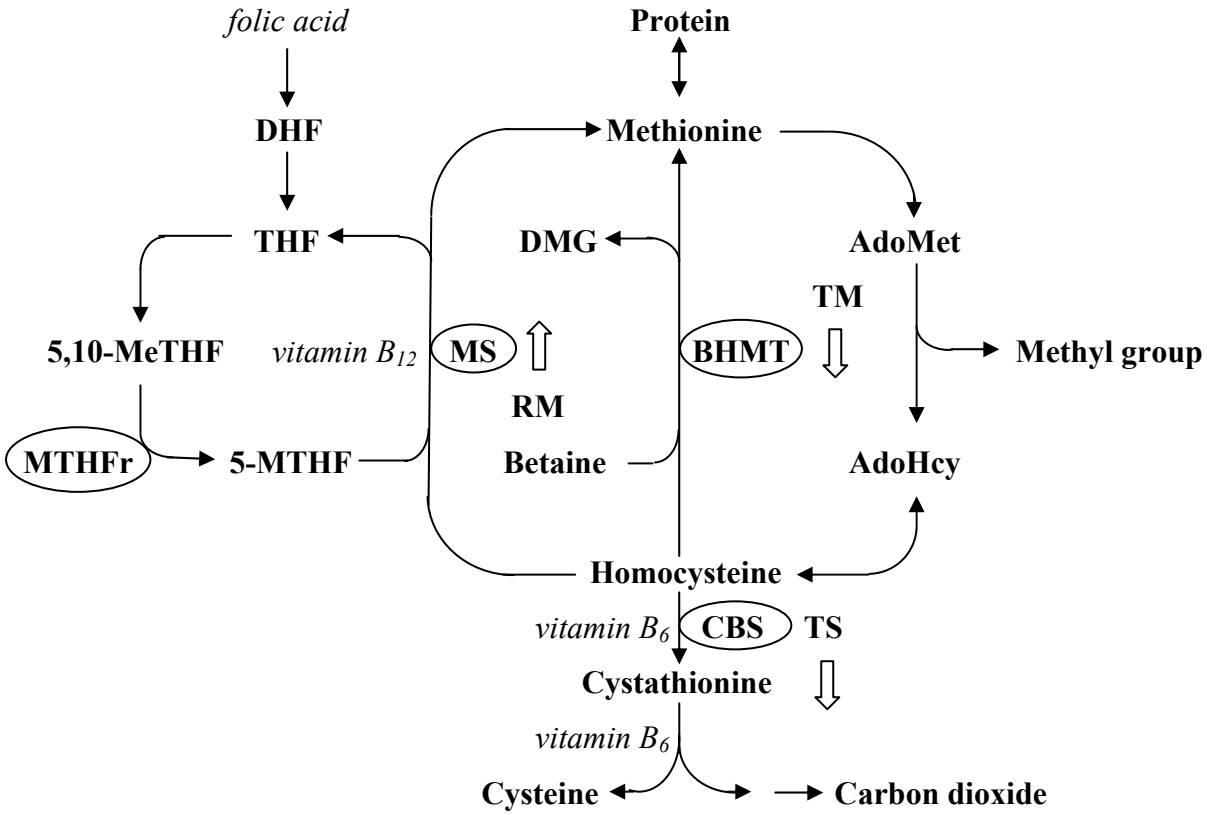

Figure 1. Pathways of homocysteine metabolism. Schematic overview of homocysteine metabolism, with open arrows indicating metabolic rates, ovals indicating enzymes, and italics indicating vitamins. RM, remethylation; TM, transmethylation; TS, transsulfuration; MTHFr, 5,10-methylenetetrahydrofolate reductase; MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; DMG, dimethylglycine; DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-MeTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate.

addition to TS, we calculated Hcy clearance as TS divided by the plasma total Hcy (tHcy) concentration.

Impaired regulation of one-carbon cycling, possibly through accumulation of uremic toxins, may be an explanation for abnormalities in RM and TM rates in ESRD patients. In patients with chronic renal failure, plasma AdoHcy and AdoMet levels are elevated [14], but methionine levels are not increased [15]. Whether and how these metabolite changes are related to tissue one-carbon fluxes has hitherto not been studied. From single-enzyme kinetics, the following interactions can be deduced [16,17]. *1*) In TS, increased levels of AdoMet and AdoHcy are activators of cystathionine β-synthase. *2*) In RM, AdoMet and AdoHcy are inhibitors of betaine-Hcy methyltransferase, and AdoHcy inhibits MS, whereas AdoMet inhibits 5,10-methylenetetrahydrofolate reductase, and both methyltransferases are inhibited by their metabolic product, methionine. *3*) In TM, AdoHcy inhibits the activity of most methyltransferases. From these data, elevated intracellular levels of AdoMet and

AdoHcy are expected to be associated with diminished RM (and TM) flux rates, whereas TS flux is expected to be stimulated by methionine ingestion and the subsequent elevations of AdoMet and AdoHcy by adenosyltransferase activity. However, little is known about the in vivo regulation of one-carbon metabolism by AdoMet and AdoHcy in healthy humans and in patients with chronic renal failure.

In view of these considerations, the aims of the present study were to test the hypothesis that in vivo one-carbon fluxes (TM, RM and TS) and the Hcy clearance by RM and TS are decreased in ESRD patients compared to healthy individuals and to explore the relationships of these fluxes with plasma Hcy and blood AdoMet and AdoHcy levels.

MATERIALS AND METHODS

Subjects

For the present study, eight ESRD patients on maintenance hemodialysis treatment and 10 healthy controls, with an age > 18 years, were included. The patients were on chronic standard bicarbonate hemodialysis (thrice weekly for at least six mo) and received a multivitamin tablet (once daily) containing 2 mg vitamin B_6, 2 mg thiamine, 2 mg riboflavin, 15 mg nicotinamide, and 70 mg ascorbic acid. Five ESRD patients received a tablet (once weekly) containing 1 mg folic acid. The healthy controls did not use any medication or vitamin supplements. To enhance the power of the study to measure a clinically relevant difference in methionine-Hcy metabolism, data from a previous study in four ESRD patients and six healthy controls were combined with those of the present study group. The results of these 10 individuals have been published previously [12]. The total study group thus consisted of 12 ESRD patients and 16 healthy individuals. The study protocol was approved by the ethics committee of the VU University Medical Center, and written informed consent was obtained from all individuals.

Experimental protocol

The experimental protocol has been described earlier [12].

Fat-free mass. Body weight (BW) was measured on a balance scale (accuracy 50 g), and four skinfolds were measured using a caliper (Holtain, accuracy 0.1 mm). Fat-free mass (FFM) was calculated from skinfold measurements according to Durnin and Womersley [18].

This method has been shown to be a reliable method of quantifying body composition in healthy individuals as well as ESRD patients [19,20].

Stable isotopes. All subjects were kept on a fixed diet containing 1.0 g of protein·kg BW^{-1}·day^{-1} for 3 days before the study. The experiments were conducted after an overnight fast. Fasting was continued throughout the infusion period. Only drinking of small amounts of tap water was allowed. The hemodialysis patients were studied 1 day before a regular midweek dialysis session. All subjects were kept in bed during the study period. A priming bolus of $NaH^{13}CO_3$ (99% ^{13}C, ARC Laboratories, Apeldoorn, The Netherlands) was administered, followed by a primed constant infusion of L-[$^2H_3$-methyl-1-^{13}C]methionine (95% dilabeled; 99% 1-^{13}C; 99% $^2H_1$, Isotec, Miamisburg, OH), according to the infusion protocol described previously [12].

Sample analysis. Plasma total (free plus protein bound) Hcy was measured with the use of a microparticle enzyme immunoassay method based on fluorescence polarization (IMX analyzer; Abbott, Chicago, IL). AdoMet and AdoHcy were measured using stable-isotope dilution tandem mass spectrometry [21]. AdoHcy and AdoMet levels were not determined in the 10 individuals described in the previous study [12]. Serum folate and vitamin B_{12} concentrations were measured in plasma and whole blood with radioassay (ICN, Costa Mesa, CA) and serum vitamin B_6 with the use of fluorescence HPLC [22]. The methionine concentration in the infusate was measured with the use of an amino acid analyzer equipped with a high-pressure analytical column packed with Utrapac 8 resin (Pharmacia Biotech, Cambridge, UK). Isotopic enrichments of methionine in plasma were measured in the acetyl-3,5-bis(trifluoromethyl)benzyl derivative with the use of gas chromatography-mass spectrometry (GCMS), as previously described [23]. Enrichments [in mole percent excess (MPE)] were calculated on the basis of the relative abundance of the (m + 0), (m + 1) and (m + 4) methionine species [24], and calibration curves obtained from weighed amounts of tracer (m + 1 and m + 4) and tracee methionine were used to correct for minor instrument variation [18]. The ^{13}C-enrichment of CO_2 in breath samples was measured on a dual-inlet isotope radio mass spectrometer (VG OPTIMA, Fisons Instruments, Middlewich, Cheshire, UK) and expressed in atom percent excess (APE; %), as previously described [12].

Gene polymorphisms. The polymorphisms of 5,10-methylenetetrahydrofolate reductase ($C_{677}T$ transition), cystathionine β-synthase (844ins68 variant) and methionine synthase ($A_{2756}G$ transition) were assessed in DNA obtained from the buffy coat of EDTA blood as described by Tsai et al. [25], who demonstrated that healthy individuals who were

heterozygous for these mutations had plasma tHcy levels that were not different from those in the wild-type subjects.

Calculations

The features of the [$^{2}H_3$-methyl-1-^{13}C]methionine plateau model have been previously summarized [24,26]. In the isotopic steady-state, whole body fluxes of TM, TS and RM can be estimated from enrichments at plateau from the five plasma samples obtained during the tracer infusion between 3.5 and 5 h. The RM flux is calculated from the difference between the whole body methionine-methyl flux rate (Q_m) and the methionine-COOH flux rate (Q_c), because the $^{2}H_3$-methyl label is split from the methionine tracer during TM, whereas ^{13}C is retained in the COOH moiety of Hcy

$$RM = Q_m - Q_c \quad (1)$$

From the infusion rate of the tracer and with correction for isotopic enrichment of the tracer, Q_m can be calculated from the enrichments of methionine (m + 4) and Q_c from the sum of the enrichments of methionine (m + 4) and (m + 1). RM then follows from substitution in Eq. 1 [12,24].

The rate of TS flux from Hcy equals the methionine oxidation flux and is calculated from the $^{13}CO_2$ (the COOH end-product of Hcy oxidation) excretion in breath air and the methionine (m + 1) enrichment in plasma and tracer infusion rate as follows

$$TS = V_{^{13}CO_2} * (1/[^{13}C]\text{methionine enrichment in plasma} - 1/[^{13}C]\text{methionine enrichment in tracer infusate}) \quad (2)$$

in which $V_{^{13}CO_2}$ equals the whole body CO_2 production (in μmol/h) * breath air $^{13}CO_2$ enrichment * 0.72, where 0.72 is the bicarbonate retention factor [27]. As methionine is the only precursor of Hcy, the disappearance of Hcy (RM + TS) equals Hcy appearance, thus

$$TM = RM + TS \quad (3)$$

CO_2 production was measured during 30 min with a ventilated hood and using an indirect calorimeter (2900 Metabolic Measurement Cart, SensorMedics, Yorba Linda, CA). Internal calibration for CO_2 and volume was conducted before each infusion. Gas volumes were

automatically corrected for temperature and air pressure. The flux rates for RM, TS and TM were expressed as micromoles per hour per kilogram BW and per kilogram FFM. Metabolic Hcy clearance by TS and by RM was calculated by dividing the flux rates (TS and RM, respectively) by plasma tHcy concentration and is expressed in liters per kilogram per hour.

Statistical analysis

Values are expressed as mean ± SD or as median (range) if data were skewed. Differences between patients and controls were tested with unpaired Student's t-tests, Mann-Whitney tests, and Fisher's exact tests as appropriate. Pearson's correlation coefficients were calculated, with log-transformed data in case of a skewed distribution of a variable. The distribution of genetic polymorphisms (wild-type or heterozygote mutant = 0; homozygote mutant = 1) was analyzed with the χ^2 test. Multivariate regression analysis was performed to analyze ESRD (absent = 0; present = 1), sex, age, and vitamin status (plasma concentrations) as determinants of FFM-adjusted flux rates and Hcy clearance. All regression coefficients (β) are reported as standardized β. Differences were considered significant if $P < 0.05$ (2-tailed).

RESULTS

The median (range) time on dialysis of the 12 ESRD patients was 17 (10–140) mo. Baseline characteristics are presented in Table 1. Patients with ESRD had significantly higher plasma levels of tHcy and folate, and a significantly higher mean age compared with the 16 healthy controls.

Whole body flux rates of RM, TM, TS, and Hcy clearance in ESRD patients and controls

CO_2 production was similar in patients (2.79 ± 0.21 $ml \cdot kg^{-1} \cdot min^{-1}$) and controls (2.72 ± 0.15 $ml \cdot kg^{-1} \cdot min^{-1}$). Patients with ESRD had a RM rate that was 28% lower than the controls [95% confidence interval (CI) –50 to –5%, $P = 0.02$], whereas TM was 27% lower (95% CI –43 to –11%, $P < 0.01$) and TS was 26% lower (95% CI –45 to –7%, $P < 0.01$). Hcy clearance by RM was 77% lower (95% CI –100 to –55%, $P < 0.001$) and Hcy clearance by TS was 80% lower (95% CI –100 to –52%, $P < 0.001$) in ESRD patients as compared with controls (Table 2).

Table 1. Baseline characteristics in ESRD patients and healthy controls

	Sex	Age, y	BW, kg	FFM, kg	MTHFr	CBS	MS	tHcy, µmol/l	Folate, nmol/l	Vit B_6, nmol/l	Vit B_{12}, pmol/l
					ESRD patients						
1, ADPKD*	M	69	81	75	CT	-/-	AG	43.6	17.8	11	182
2, HUS*	F	51	82	62	CC	-/-	AG	63.2	17.4	93	403
3, CGN*	F	31	61	48	CT	-/-	AA	49.4	14.3	32	193
4, CGN*	F	25	54	42	CC	-/+	AG	57.0	14.2	16	201
5, ADPKD	M	63	75	62	CT	-/-	AG	37.9	21.2	53	464
6, FSGS	F	52	109	68	CC	-/-	AA	27.5	14.9	47	431
7, RAS	M	53	74	60	CC	-/-	AA	16.6	28.0	14	385
8, PRO	M	69	78	66	TT	-/-	GG	19.6	45.0†	42	213
9, HN	M	79	67	58	CT	-/-	AG	28.1	45.0†	89	302
10, MPGN	M	60	59	54	CT	-/-	AG	16.6	45.0†	61	336
11, HN	M	24	63	55	CT	-/+	AG	14.8	45.0†	48	573
12, PRO	M	70	76	64	CT	-/-	AA	26.7	15.2†	47	218
Mean ± SD or median (range)	8 M 4 F	54 ± 18	73 ± 14	59 ± 9	4 CC 7 CT 1 TT	10 -/- 2 -/+ 0 +/+	4 AA 7 AG 1 GG	33.4 ± 16.6	27.8 (14.2–45.0)	47 (11–93)	319 (182–573)
					Healthy controls						
*1**	M	41	68	62	CC	-/-	GG	7.0	16.1	44	290
*2**	M	20	70	60	CT	-/-	AG	5.6	28.7	81	368
*3**	M	22	78	66	TT	-/-	AG	14.1	11.7	35	186
*4**	M	50	84	69	CT	-/+	AA	7.7	14.1	58	332
*5**	F	21	75	60	CC	-/-	AA	10.2	12.3	70	124
*6**	F	53	63	47	CT	-/-	AG	12.0	23.0	27	197
7	M	35	74	65	CC	-/-	AA	9.6	12.9	26	208
8	F	22	62	44	CC	-/-	AA	5.6	16.2	28	274
9	F	23	64	47	CC	-/-	AA	10.6	7.3	20	260
10	M	22	73	61	CC	-/+	AA	8.9	17.2	29	216
11	F	19	60	40	CT	-/-	AA	7.0	18.7	38	175
12	M	20	69	57	CC	-/-	AG	6.1	14.6	44	214
13	F	19	66	46	CC	-/-	AA	7.1	15.4	55	181
14	F	47	57	43	CT	-/+	AA	7.4	10.4	20	313
15	M	64	108	90	CC	-/-	AA	8.6	15.1	24	227
16	F	46	63	46	CT	-/-	AA	7.4	17.5	8	218
Mean ± SD or median (range)	8 M 8 F	32 ± 16	71 ± 12	56 ± 13	9 CC 6 CT 1 TT	13 -/- 3 -/+ 0 +/+	11 AA 4 AG 1 GG	8.4 ± 2.4	15.3 (7.3–28.7)	32 (8–81)	217 (124–368)
P value‡	0.21	<0.01	0.65	0.50	1.00	1.00	1.00	<0.001	0.02	0.38	0.07

M, male; F female; BW, body weight; FFM, fat-free mass; MTHFr, 5,10-methylenetetrahydrofolate reductase; CBS, cystathionine β-synthase; MS, methionine synthase; CC, wildtype; CT, heterozygous for $C_{677}T$; TT, homozygous for $C_{677}T$; -/-, wildtype; -/+, heterozygous for 844ins68; +/+, homozygous for 844ins68; AA, wildtype; AG, heterozygous for $A_{2756}G$; GG, homozygous for $A_{2756}G$; tHcy, total plasma homocysteine; Vit, vitamin; ESRD, end-stage renal disease; ADPKD, autosomal dominant polycystic kidney disease; HUS, hemolytic uremic syndrome; CGN, chronic glomerulonephritis; FSGS, focal and segmental glomerulosclerosis; RAS, renal artery stenosis; PRO, postrenal obstruction; HN, hypertensive nephropathy; MPGN, membranoproliferative glomerulonephritis. *Data from previous study group presented by Van Guldener et al. [12]; FFM and genotype analysis for CBS and MS are added. †Patients with additional use of 1 mg folic acid once weekly; the upper limit of the folate assay was 45 nmol/l. ‡Comparison of the group patients with ESRD with the group of healthy controls.

RM and TM rates were positively correlated, both within the patient group ($r = 0.84$, $P < 0.001$) and within the control group ($r = 0.89$, $P < 0.001$). Correlations between RM and TS and between TM and TS were not significant in either group (data not shown).

After adjustment for sex and age, RM (–18%; 95% CI –45 to +9%, P = 0.19), TM (–15%; 95% CI –33 to +2%, P = 0.09), and TS (–10%; 95% CI –30 to +9%, P = 0.29) were no longer significantly decreased in the ESRD group, but Hcy clearance by RM (–65%; 95% CI –97 to

Table 2. Rates of methionine turnover and homocysteine metabolism in the postabsorptive state, expressed per kilogram BW or FFM

	RM, mmol·kg^{-1}·h^{-1}		RM/tHcy, l·kg^{-1}·h^{-1}		TS, mmol·kg^{-1}·h^{-1}		TS/tHcy, l·kg^{-1}·h^{-1}		TM, mmol·kg^{-1}·h^{-1}	
	BW	FFM	BW	FFM	BW	FFM	BW	FFM	BW	FFM
					ESRD patients					
*1**	2.1	2.3	0.05	0.05	1.7	1.9	0.04	0.04	3.9	4.2
*2**	2.8	3.8	0.04	0.06	3.0	4.0	0.05	0.06	5.8	7.8
*3**	2.6	3.3	0.05	0.07	3.0	3.9	0.06	0.08	5.6	7.2
*4**	2.9	3.7	0.05	0.06	2.4	3.1	0.04	0.05	5.3	6.8
5	3.1	3.7	0.08	0.10	2.3	2.8	0.06	0.07	5.4	6.5
6	2.3	3.7	0.08	0.13	1.4	2.3	0.05	0.08	3.7	5.9
7	5.3	6.5	0.32	0.39	2.4	3.0	0.15	0.18	7.7	9.5
8	3.4	4.0	0.17	0.20	1.5	1.8	0.08	0.09	4.8	5.7
9	3.9	4.5	0.14	0.16	1.8	2.1	0.06	0.07	5.6	6.5
10	2.7	3.0	0.16	0.18	1.6	1.7	0.10	0.11	4.2	4.6
11	4.1	4.7	0.28	0.32	2.7	3.1	0.18	0.21	6.8	7.8
12	2.3	2.8	0.09	0.10	2.4	2.9	0.09	0.11	4.7	5.6
Mean ± SD	3.1 ± 0.9	3.8 ± 1.1	0.13 ± 0.09	0.15 ± 0.11	2.2 ± 0.6	2.7 ± 0.8	0.08 ± 0.04	0.10 ± 0.05	5.3 ± 1.2	6.5 ± 1.5
					Healthy controls					
*1**	4.0	4.4	0.57	0.63	3.2	3.5	0.46	0.50	7.2	7.9
*2**	5.2	6.1	0.93	1.09	3.0	3.5	0.54	0.63	8.2	9.6
*3**	3.9	4.6	0.28	0.33	2.8	3.3	0.20	0.23	6.7	7.9
*4**	3.0	3.6	0.39	0.47	3.2	3.9	0.42	0.50	6.2	7.5
*5**	3.1	3.9	0.30	0.38	3.0	3.8	0.29	0.37	6.0	7.5
*6**	3.3	4.4	0.27	0.37	2.8	3.7	0.23	0.31	6.2	8.3
7	3.7	4.2	0.39	0.44	2.0	2.2	0.21	0.23	5.7	6.4
8	3.2	4.5	0.57	0.81	3.4	4.9	0.61	0.87	6.6	9.4
9	5.4	7.3	0.51	0.69	2.3	3.1	0.22	0.29	7.7	10.5
10	4.7	5.6	0.53	0.63	3.4	4.0	0.38	0.45	8.0	9.6
11	4.4	6.6	0.63	0.95	3.1	4.6	0.44	0.66	7.5	11.2
12	3.9	4.7	0.64	0.78	4.6	5.5	0.75	0.91	8.5	10.3
13	7.0	10.1	0.99	1.42	2.8	4.1	0.40	0.58	9.8	14.2
14	2.9	3.9	0.40	0.52	3.2	4.2	0.43	0.57	6.1	8.1
15	5.7	6.9	0.66	0.80	1.9	2.3	0.22	0.27	7.6	9.2
16	2.5	3.4	0.34	0.46	1.5	2.1	0.20	0.28	4.0	5.5
Mean ± SD	4.1 ± 1.2	5.3 ± 1.8	0.52 ± 0.21	0.67 ± 0.30	2.9 ± 0.7	3.7 ± 0.9	0.37 ± 0.16	0.48 ± 0.21	7.0 ± 1.4	8.9 ± 2.1
P value†	0.02	0.02	< 0.001	< 0.001	0.01	< 0.01	< 0.001	< 0.001	< 0.01	< 0.01

RM, remethylation; TS, transsulfuration; TM, transmethylation; tHcy, plasma total homocysteine. *Data for BW from previous study by Van Guldener et al. [12]; corrections for FFM are added. †Comparison of patients with ESRD with healthy controls.

–32%, P < 0.001) and Hcy clearance by TS (–65%; 95% CI –97 to –33%, P < 0.001) were still significantly decreased. Age itself was a significant determinant of TS (β = –0.51, P = 0.01) and TM (β = –0.45, P = 0.03), but not of RM (β = –0.29, P = 0.21), Hcy clearance by RM (β = –0.25, P = 0.14) and Hcy clearance by TS (β = –0.29, P = 0.08). Plasma tHcy, the other component of the equation for Hcy clearance by TS and RM, was not significantly determined by age (β = –0.04, P = 0.80).

Because ESRD status and age were strongly associated, analyses adjusted for age may to some extent result in overadjustment. We therefore repeated the multivariate analysis in two age-matched subgroups, which consisted of 8 ESRD patients (patients 2, 3, 4, 5, 6, 7, 10, and 11; mean age 44.9 ± 15.7 yr) and 8 healthy controls (patients 1, 3, 4, 6, 9, 14, 15, and 16; mean age 43.3 ± 14.4 years). After adjustment for sex and age, the ESRD group had a 23% lower RM (95% CI –44 to –3%, P = 0.03), a 19% lower TM (95% CI –38 to –1%, P = 0.048), a 72% lower Hcy clearance by RM (95% CI –96% to –49%, P < 0.001), and a 72% lower Hcy

clearance by TS (95% CI –100 to –41%, $P < 0.001$) compared with the healthy controls, but TS was not significantly different (–8%; 95% CI –33 to +17%, $P = 0.51$).

Separate addition of serum levels of vitamins to the statistical models did not change the results (data not shown).

Determinants of plasma tHcy

Plasma tHcy was about four-fold higher in the ESRD group than in the healthy controls.

Multivariate analysis, with forced entry of the covariates sex and age, showed that patients with ESRD had a 322% higher plasma tHcy (95% CI 209 to 436%, $P < 0.001$). To test the possibility that this relation was mediated by one-carbon flux rates, plasma and whole blood levels of AdoMet and AdoHcy (and their ratio), and vitamins, these individual variables were sequentially added to the multivariate model. This did not materially change the relationship between ESRD status and plasma tHcy. After adjustment for age and sex, RM ($\beta = –0.37$, $P = 0.07$) and TM ($\beta = –0.42$, $P = 0.08$) were associated with plasma tHcy with borderline significance, and TS ($\beta = –0.08$, $P = 0.75$) was not. Plasma levels of AdoMet ($\beta = 0.78$, $P < 0.001$), AdoHcy ($\beta = 0.74$, $P < 0.001$), and their ratio ($\beta = -0.69$, $P = 0.001$) were related to plasma tHcy, as were whole blood levels of AdoHcy ($\beta = 0.63$, $P < 0.01$) and the AdoMet/Hcy ratio ($\beta = –0.55$, $P = 0.01$), but whole blood levels of AdoMet were not significantly related to plasma tHcy ($\beta = –0.15$, $P = 0.46$). Of the vitamins, serum folate was significantly related to plasma tHcy ($\beta = –0.38$, $P = 0.01$), but vitamin B_6 ($\beta = –0.12$, $P = 0.63$) and vitamin B_{12} ($\beta = –0.16$, $P = 0.24$) were not.

Blood AdoMet and AdoHcy and their relationship to flux rates and Hcy clearance

Table 3 shows the levels of AdoMet, AdoHcy, and their ratio in whole blood and plasma for patients with ESRD and healthy controls. After adjustment for sex and age, plasma levels of AdoMet ($\beta = 0.87$, $P < 0.001$) and AdoHcy ($\beta = 0.97$, $P < 0.001$) were significantly higher in the ESRD patients compared with the healthy controls, and the AdoMet/AdoHcy ratio was lower ($\beta = –1.00$, $P < 0.001$). In whole blood, the same results were found for AdoHcy ($\beta = 0.95$, $P < 0.001$) and the AdoMet/AdoHcy ratio ($\beta = –1.01$, $P < 0.001$), but AdoMet levels of the ESRD patients were not significantly different ($\beta = –0.59$, $P = 0.08$) from the healthy controls.

After adjustment for sex and age, whole blood levels of AdoHcy were a significant determinant of RM ($\beta = –1.43$, $P = 0.03$), TM ($\beta = –1.24$, $P = 0.01$) (Figure 2), but not of TS ($\beta = –0.37$, $P = 0.48$), Hcy clearance by RM ($\beta = –0.55$, $P = 0.23$), and Hcy clearance by TS

Table 3. Plasma and whole blood levels of S-adenosylmethionine and S-adenosylhomocysteine in the postabsorptive state

	AdoMet, nmol/l		AdoHcy, nmol/l		AdoMet/AdoHcy		
	Plasma	Whole blood	Plasma	Whole blood	Plasma	Whole blood	Hematocrit
			ESRD patients				
5	NA	NA	NA	NA	NA	NA	0.38
6	268	1435	252	262	1.1	5.5	0.41
7	190	1195	139	124	1.4	9.6	0.34
8	390	1260	562	407	0.7	3.1	0.32
9	304	1610	325	230	0.9	7.0	0.36
10	247	1150	410	294	0.6	3.9	0.38
11	240	1120	437	374	0.6	3.0	0.32
12	346	1350	465	305	0.7	4.4	0.37
Mean ± SD or median (range)	268 (190–390)	1260 (1120–1610)	410 (139–562)	294 (124–407)	0.7 (0.6–1.4)	4.4 (3.0–9.6)	0.36 ± 0.03
			Healthy controls				
7	70	NA	11	NA	6.4	NA	0.41
8	61	1335	10	77	5.9	17.3	0.36
9	74	1095	13	53	5.7	20.7	0.35
10	83	1760	13	70	6.6	25.1	0.42
11	102	1580	13	62	7.9	25.4	0.37
12	87	1615	11	66	8.3	24.7	0.38
13	88	1360	12	58	7.1	23.4	0.33
14	91	1630	9	63	10.4	26.1	0.37
15	89	1660	13	57	6.7	29.0	0.42
16	81	1220	12	88	6.8	13.9	0.37
Mean ± SD or median (range)	85 (61–102)	1580 (1095–1760)	12 (9–13)	63 (53–88)	6.7 (5.7–10.4)	24.7 (13.9–26.1)	0.38 ± 0.03
P value*	< 0.001	0.06	< 0.001	< 0.001	< 0.001	< 0.001	0.20

AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; NA, not available. *Comparison of patients with ESRD with healthy controls.

(β = 0.02, P = 0.97) in the whole group. The whole blood levels of AdoMet were significantly associated with TS (β = 0.40, P = 0.049), but not with RM (β = –0.06, P = 0.85), TM (β = 0.14, P = 0.57), Hcy clearance by RM (β = 0.02, P = 0.92), or Hcy clearance by TS (β = 0.25, P = 0.20). The whole blood AdoMet/AdoHcy ratio was significantly associated with TM (β = 0.51, P = 0.02), and less with RM (β = 1.27, P = 0.07) and TS (β = 0.68, P = 0.15). The whole blood AdoMet/AdoHcy ratio was not a significant determinant of Hcy clearance by RM (β = 0.49, P = 0.27) and Hcy clearance by TS (β = 0.22, P = 0.63). Plasma levels of AdoMet, AdoHcy or the AdoMet/AdoHcy ratio were not significantly associated with one-carbon fluxes or Hcy clearance (data not shown).

Data were insufficient to test whether the relationship between ESRD status on the one hand and one-carbon fluxes and Hcy clearances on the other were mediated by AdoMet, AdoHcy, or their ratio.

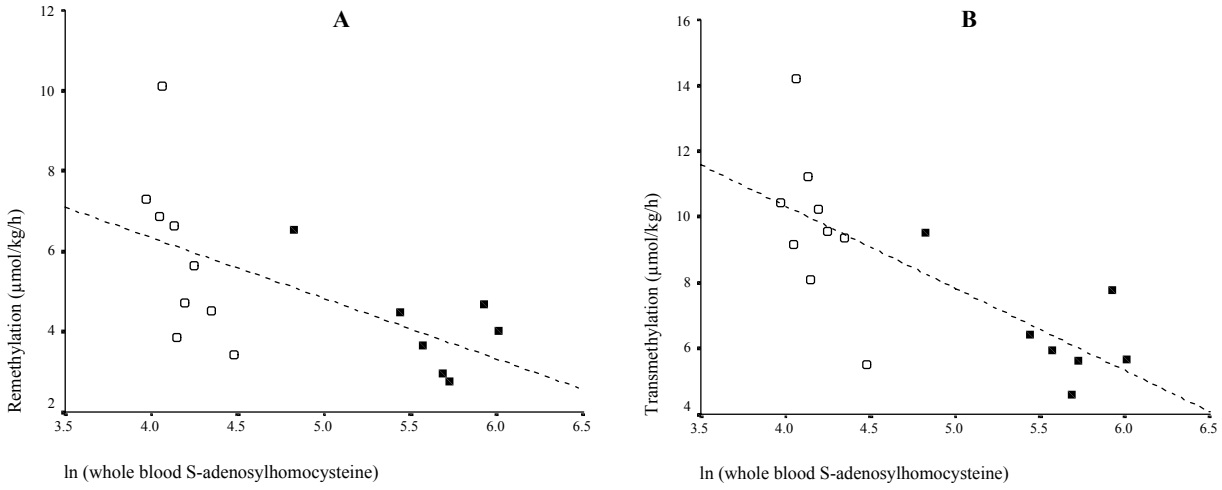

Figure 2. Relation between whole blood S-AdoHcy levels and 1-carbon flux rates in 7 patients with end-stage renal disease (■) and 9 healthy controls (o). Whole blood S-AdoHcy is expressed as the natural logarithm (ln) of the concentration (in nmol/l), and RM and TM fluxes are expressed per kg fat-free mass. The correlation coefficient (r) in **A** (RM) for the whole group is –0.61 ($P < 0.01$); –0.68 ($P = 0.09$) for end-stage renal disease; and –0.65 ($P = 0.11$) for controls. In **B**, r for whole group TM is –0.75 ($P < 0.001$); –0.71 ($P = 0.03$) for end-stage renal disease; and –0.66 ($P = 0.06$) for controls. Dotted lines depict logarithmic regression lines for all individuals and are added for illustrative purposes.

DISCUSSION

Our study shows that in ESRD patients, metabolic Hcy clearance by TS and RM is severely reduced and indicates that absolute RM and TM rates are reduced to a much lesser extent, whereas the TS rate is unchanged. We also found evidence for an inverse relationship between whole blood AdoHcy concentration and TM and RM rates.

In steady-state conditions in weight-maintaining adults, TS rate reflects oxidation of methionine from dietary intake, because methionine is the only precursor of Hcy, and TS is the only way of Hcy disposal (methionine catabolism by transamination is negligible in humans) [28]. In the design of our study, protein intake was the same in ESRD patients and healthy controls. It is thus not surprising that in the present study TS rate (after adjustment for age and gender) was similar for ESRD patients and controls. This is in agreement with observations in our previous study [12].

As an explanation of the elevated plasma tHcy concentrations in ESRD patients, the concept of decreased metabolic Hcy clearance has been put forward [13]. In this concept, any block in Hcy transsulfuration has to be followed by an increase in plasma tHcy in order to maintain a constant (diet-dependent) methionine oxidation. The initial rise in Hcy is followed

by an accumulation of its precursors AdoHcy and AdoMet, which are activators of cystathionine β-synthase [16,29], the rate limiting enzyme in the TS pathway. The rise in the concentration of these compounds in ESRD may stimulate TS to such an extent that it equilibrates with methionine intake. We and others [14] found elevated AdoMet and AdoHcy levels in ESRD patients, and we also found that whole blood levels of AdoMet were positively related to TS. Thus in the steady state, the absolute TS rate reflects oxidation of ingested methionine, but the ratio of TS and plasma tHcy (i.e., Hcy clearance by TS) reflects the degree of the impairment of TS to metabolize its substrate. Expressed in this way, Hcy clearance was decreased by 80% in ESRD patients, which is in good agreement with the reduction in plasma Hcy clearance by 75% reported after oral Hcy loading in patients with chronic renal failure [5]. From our study, the cause of this metabolic block cannot be determined.

The interpretation of the whole body RM rate in ESRD patients is somewhat difficult as these patients were older than the control individuals. After correction for age, there was no significant difference in RM, but because age and renal function are strongly related there might have been some overcorrection. To prevent such a statistical effect, we also performed an age-matched subgroup analysis and, by doing so, found a significantly lower RM in the renal patients. However, even in the presence of a normal absolute RM flux, an elevated plasma tHcy level would be indicative of a metabolic RM block under the metabolic clearance concept. Substantial hyperhomocysteinemia is common in ESRD [1] and was present in all patients participating in our study. This is evidence for a substantial metabolic block in Hcy clearance by RM in the majority of the ESRD population. Treatment regimens containing folic acid decrease plasma tHcy concentration in ESRD patients [2–4], which indicates that this block is modifiable.

TM was diminished in ESRD patients, despite accumulation of the methyl group donor AdoMet. However, in accordance with Loehrer and co-workers [14], we found that the ratio of AdoMet to AdoHcy in plasma was eight times lower in ESRD patients than in healthy individuals. From in vitro studies, the AdoMet/AdoHcy ratio is known to have a regulatory function in the enzymatic TM reactions [16]. The metabolic consequence of diminished whole body TM flux in ESRD patients is impaired capacity to provide methyl groups in tissues. This may explain the impaired DNA methylation in hyperhomocysteinemic ESRD patients as recently reported [30]. Less requirement for creatine biosynthesis, and therefore methyl donation, due to diminished muscle mass in ESRD patients has been suggested to result in a smaller need for TM (and RM) by Hoffer [13]. However, in our study we expressed

TM per kilogram FFM, which was similar in healthy controls and ESRD patients. Whether reducing tissue AdoHcy accumulation can increase methylation flux rates in patients with ESRD and will improve DNA methylation merits further study.

This is the first study in humans that shows that the whole body flux rates of RM and TM are negatively related to the whole blood AdoHcy level. The inverse relation between AdoHcy levels and RM rate may be explained by inhibitory effects of AdoHcy on methionine synthase activity in the tissues and most other acceptor methyltransferases, as suggested by Finkelstein [16]. Also, in our study design, protein intake was fixed and TS unchanged, implying that RM and TM are linearly related with each other (see Eq. 3). Therefore, RM and TM showed the same trend in their relationships with AdoHcy. The whole blood AdoMet/AdoHcy ratio showed only a weak relationship with TM and none with RM, and plasma AdoHcy was not related to RM and TM flux. This suggests that the intracellular AdoHcy concentration, reflected by its whole blood levels, is a possible regulator of methyl group flux from methionine demethylation and folate-dependent RM in humans. Plasma tHcy was not significantly associated with RM or TM in our ESRD and healthy control individuals. Taken together, these findings indicate that in humans, and particularly when renal function is impaired, AdoHcy may be a key intracellular regulator of one-carbon flux.

The limitations of the study need to be addressed. First, in retrospect, the present study had a power of 80% to detect a difference of 27% in TS and TM and 38% in RM, but, despite inclusion of data from our previous stable-isotope study [12], the number of subjects was too small to quantify the impact of AdoHcy and AdoMet on the relationship between ESRD and flux rates. The conclusions regarding relationships have to be interpreted with caution, as we cannot exclude that other factors associated with ESRD (e.g., uremic compounds) interact with enzyme function in methionine and Hcy metabolism.

Second, the validity of the stable-isotope model as a reflection of intracellular methyl group metabolism merits discussion. Isotope retention in the body's bicarbonate pool is a recognized cause of underestimation of catabolism when $^{13}CO_2$ enrichment in breath air is used, and therefore TS flux was corrected with the bicarbonate retention factor [31]. Also, RM, TM, and TS fluxes are underestimated to some extent when plasma enrichments are used in the calculations, due to tracer dilution in the intracellular compartment. Young and co-workers [24] assumed, from measurements of dilution of other indispensable amino acids, that use of plasma values underestimates RM and TM in postabsorptive adults by ~20%. In labeled methionine studies in humans, enrichment in plasma Hcy, a surrogate measure of intracellular methionine enrichment, was 90% of tracer methionine enrichment in plasma after 9 h [32].

Lower values for plasma enrichment of Hcy (the precursor for RM) compared with methionine have been found in studies using priming of the methionine pool [33,34], because, in the absence of priming of the Hcy pool, plasma enrichment rises much more slowly than when priming is used. There are no known pathways for Hcy production apart from TM, rendering additional dilution of intracellular Hcy unlikely and underpinning the validity of the use of [^{13}C]methionine enrichment for methionine and Hcy flux estimation. With the use of our study protocol, plasma methionine enrichment reaches plateau in primed steady-state protocols within 6 h, even in ESRD patients [23], in whom hyperhomocysteinemia coexists with normal methionine levels [15]. With these considerations, we do not expect the underestimation of TM and RM in ESRD patients and healthy controls to be much different from 10–20% under the conditions of our study.

Third, with respect to the role of selection bias on interpretation of the study data, the effect of age and vitamin supplementation must be addressed. There was a difference of ~22 yr in mean age between the ESRD patients and healthy controls in our study, and multiple regression analysis showed a significant negative relationship between their age and TS and TM rates. With the same stable-isotope infusion technique, others have reported similar flux rates in whole body methionine and Hcy metabolism in healthy elderly (n = 12; age 74 ± 2 yr) and young individuals (n = 8; age 27 ± 6 yr) on the same protein intake [31,35]. In post hoc analysis, ESRD patients had a significantly lower TM and RM than age-matched controls, but TS was similar. The patient-control difference in TS rate between the post hoc analysis (–8%) and the overall analysis (–27%) cannot be easily explained entirely by age alone. A difference in vitamin intake is another potential bias. Five ESRD patients received low-dose folic acid supplementation (1 mg once weekly). Compared with the other seven ESRD patients, these supplemented patients had significantly lower plasma tHcy levels (21.2 ± 6.0 versus 42.2 ± 16.3 μmol/l, P = 0.02), but similar rates of RM (3.8 ± 0.9 vs. 3.8 ± 1.3 μmol·kg FFM^{-1}·h^{-1}, *P* = 0.92) and the other fluxes (data not shown). When plasma folate levels were used in multivariate analysis, to correct for confounding by folic acid substitution, no significant changes in outcome were found. No conclusions on whether or to which extent folic acid supplementation may increase the rate of the folate-dependent RM pathway in ESRD can be drawn from the data of the present study.

In summary, this study shows that one-carbon metabolism in patients with ESRD is characterized by impaired in vivo metabolic Hcy clearance by TS and RM. Elevated levels of AdoHcy, AdoMet, or both stimulate TS until the flux rate equals methionine intake from the diet. Our data suggest that the absolute rates of RM and TM are diminished and are negatively

related to whole blood AdoHcy levels. We hypothesize that the elevated intracellular concentration of AdoHcy in ESRD mediates the impairment in RM and TM flux rates. Given the persistence of hyperhomocysteinemia in ESRD patients supplemented with high doses of folic acid, the development of methods to normalize TM by reducing AdoHcy levels is of major interest.

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Chapter 4

Effect of folic acid on methionine and homocysteine metabolism in end-stage renal disease

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ABSTRACT

Background. The pathogenesis of hyperhomocysteinemia in end-stage renal disease (ESRD) is unclear. Folic acid lowers, but does not normalize, the plasma homocysteine level in patients with ESRD, but its effect on whole body metabolism of homocysteine is unknown.

Methods. We studied the effect of 3 weeks of oral treatment with 5 mg folic acid per day on homocysteine metabolism in six chronic hemodialysis patients and six healthy controls. Primed, continuous infusions with [$^{2}H_3$-methyl-1-^{13}C] methionine were used to determine flux rates of methionine transmethylation, homocysteine remethylation, and homocysteine transsulfuration. Metabolic homocysteine clearance was defined as the ratio of transsulfuration and plasma homocysteine level.

Results. Folic acid treatment lowered plasma homocysteine significantly by 48% (95% CI 8 to 88) in the ESRD group, but plasma homocysteine remained higher than baseline values in the control group. In ESRD patients, homocysteine remethylation and methionine transmethylation rate increased by 32% (95% CI 5 to 58) and 20% (95% CI 8 to 32), respectively (i.e., levels that were similar to the baseline values of the control group). Transsulfuration rate and metabolic homocysteine clearance were not significantly altered by folic acid treatment in both the ESRD and the control group.

Conclusions. In ESRD patients, folic acid treatment lowers, but does not normalize plasma homocysteine, whereas homocysteine remethylation and methionine transmethylation increase to levels found in untreated healthy controls. These findings indicate a persistent, folate-independent, defect in metabolic homocysteine clearance in ESRD.

INTRODUCTION

Hyperhomocysteinemia is associated with cardiovascular disease [1], and is a prevalent risk factor for cardiovascular disease in patients with chronic renal insufficiency [2]. Hyperhomocysteinemia occurs in 85 to 100% of patients with end-stage renal disease (ESRD) [3]. The pathophysiological explanation for the strong inverse relationship between renal function and plasma homocysteine level is controversial and focuses on whether the defect in homocysteine clearance is located in the kidneys themselves or should be sought extrarenally [4,5]. We have studied whole body metabolism of homocysteine in patients with ESRD by applying stable isotope methods [6]. In this model, primed, continuous infusions with bilabeled methionine were used to determine flux rates of methionine transmethylation to homocysteine, homocysteine remethylation to methionine and homocysteine transsulfuration to cysteine (Figure 1). We found that transmethylation and remethylation were reduced in hyperhomocysteinemic ESRD patients compared to healthy individuals. Transsulfuration was similar, reflecting the same protein (methionine) intake in both groups. The metabolic clearance of homocysteine by transsulfuration, which is expressed by the ratio of transsulfuration and plasma homocysteine [7], however, was severely impaired in ESRD patients when compared to healthy individuals [8].

Several homocysteine-lowering treatments have been tested in ESRD patients [9]. There is consensus that folic acid is the cornerstone of any homocysteine-lowering regimen in this patient group; the lowest effective dose seems to be around 1 mg daily. However, studies on

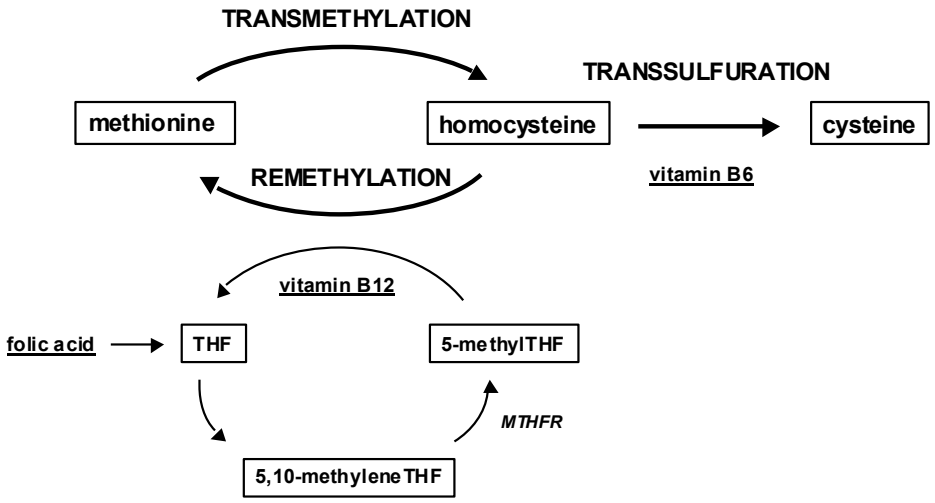

Figure 1. Schematic overview of the homocysteine metabolism. Abbreviations are: THF, tetrahydrofolate; MTHFr, 5,10-methylenetetrahydrofolate reductase.

treatment with folic acid in dialysis patients have shown that normalization of plasma homocysteine is seldom reached, which has led some authors to suggest that this folic acid resistance is the result of an altered folate metabolism in renal failure [10]. These claims have so far not been sustained by studies using active folate compounds instead of folic acid or intravenous administration of folinic acid [11–13]. In addition, it is not clear from these studies whether folic acid in ESRD patients is capable of restoring whole body homocysteine metabolism to normal values.

In this study, we aimed to elucidate the apparent folate resistance in ESRD by investigating the effect of folic acid administration on plasma homocysteine and whole body methionine and homocysteine conversion rates in ESRD patients using the tracer infusion technique with doubly labeled methionine.

METHODS

Six hemodialysis patients and six healthy control individuals were studied. Renal diagnoses were chronic glomerulonephritis in two, polycystic disease in two, hypertensive renovascular disease in one and focal glomerulosclerosis in one. All patients had been on chronic standard bicarbonate hemodialysis thrice weekly for at least 6 months. They received one multivitamin tablet per day containing 2 mg vitamin B_6, but no folic acid or vitamin B_{12}. Table 1 shows other baseline characteristics.

Table 1. Baseline characteristics in six patients with end-stage renal disease (ESRD) and six healthy controls

Characteristic	Control	ESRD
Sex, *male/female*	3 / 3	3 / 3
Age, *years*	50 ± 9	49 ± 17
Time on dialysis, *months*		22 ± 9
Body weight, *kg*	76 ± 21	76 ± 19
Fat free mass, *kg*	62 ± 19	59 ± 12
Homocysteine, *µmol/L*	8.1 ± 1.4	38.7 ± 14.8*
Folate, *nmol/L*	15 (10–18)	18 (14–28)
Vitamin B_6, *nmol/L*	24 (8–58)	21 (11–53)
Vitamin B_{12}, *pmol/L*	290 (218–332)	293 (182–464)

Values are presented as mean ± SD for variables with a normal distribution or median (range) for variables with a skewed distribution. *$P < 0.01$.

The study protocol was approved by the ethics committee of the VU University Medical Center, and all participants gave their written informed consent.

Experimental protocol

The study protocol has been described in detail elsewhere [6]. Briefly, the participants were kept on a stable protein diet of 1.0 g/kg/day. In the hemodialysis patients, the experiments were executed the day between two regular midweek dialysis sessions. All individuals remained fasting and recumbent during the test. After baseline samples were taken, a priming bolus of 5.9 μmol $NaH^{13}CO_3$ (99% [^{13}C]) (ARC Laboratories, Apeldoorn, The Netherlands) was administered, followed by a primed (3.5 μmol/kg) constant infusion of L-[$^2H_3$-methyl-1-^{13}C]methionine (95% doubly labeled) (99% [1-^{13}C]; 99% [$^2H_1$]) (Isotec, Miamisburg, OH, USA) at a rate of 2.2 μmol/kg/hour for 6 hours (5 hours in control individuals). Plateau enrichment levels were calculated as the mean of the final five 20-minute interval samples of the infusion period. Body weight was measured on a balance scale (accuracy 50 g), and four skinfolds were measured using a caliper (Holtain) (accuracy 0.1 mm). Fat-free mass was calculated from skinfold measurements according to Durnin and Womersley [14]. Measurement of skinfold thicknesses has shown to be a reliable method to quantify body composition in healthy individuals and ESRD patients [15].

Intervention

The study protocol was repeated after 3 weeks of oral treatment with 5 mg folic acid once a day.

Laboratory analyses

Plasma total (free plus protein bound) homocysteine was measured with the use of a microparticle enzyme immunoassay method based on fluorescence polarization (IMX analyzer) (Abbott, Chicago, USA). Serum folate and vitamin B_{12} concentrations were measured by radioassay (ICN, Costa Mesa, CA, USA), and serum vitamin B_6 with the use of fluorescence high-performance liquid chromatography (HPLC) [16]. The methionine concentration in the infusate was measured with an amino acid analyzer equipped with a high pressure analytical column packed with Utrapac 8 resin (Pharmacia Biotech, Cambridge, UK). Isotopic enrichments of methionine in plasma was measured in the acetyl-3,5-bis(trifluoromethyl)benzyl derivative with the use of gas chromatography mass spectrometry (GCMS), as previously described [17]. Enrichments [in mole percent enrichment (MPE)] were calculated on the basis of the abundance relative of the (m + 0), (m + 1), and (m + 4) methionine species [18], and calibration curves obtained from weighed amounts of tracer (m + 1 and m + 4) and tracee methionine were used to correct for minor instrument variation

[17]. The ^{13}C-enrichment of carbon dioxide in breath samples was measured on a dual-inlet isotope ratio mass spectrometer (VG OPTIMA) (Fisons Instruments, Middlewich, Cheshire, UK) and expressed in atom percent excess (APE) (%).

Calculations

Doubly labeled methionine (L-[$^{2}H_3$-methyl-1-^{13}C]methionine) was used as a tracer, according to the method described by Storch et al. [18]. This stable isotope has a molecular weight of m + 4 relative to natural methionine (m). The $^{2}H_3$-methyl label is removed from methionine during transmethylation and thus $^{2}H_3$-methyl-1-^{13}C]methionine is converted to [1-^{13}C]homocysteine. Remethylation will result in the generation of m + 1 methionine, because the ^{13}C atom of the carboxyl moiety of homocysteine remains intact. In contrast, during transsulfuration the carboxyl moiety of [1-^{13}C]homocysteine loses its ^{13}C atom. When α-ketobutyrate is oxidized in the Krebs cycle, the label ultimately appears as $^{13}CO_2$ in breath air. The m + 4 methionine tracer is diluted by methionine entering the pool via the diet, from homocysteine remethylation, and by free methionine entering from protein breakdown in the tissues. In steady state, the rate of appearance of methionine from these sources equals the rate of disappearance (i.e., protein synthesis and transmethylation). In this model, it is assumed that the intracellular and intravascular compartments are in rapid and complete isotopic equilibrium.

From the enrichments of methionine (m + 4 and m + 1), the whole body methionine-methyl rate of appearance and disappearance (Q_m) and methionine-carboxyl rate of appearance and disappearance (Q_c) are calculated as follows:

$$Q_m = I * (E_{tr} / E_4 - 1)$$
$$Q_c = I * (E_{tr} / (E_1 + E_4) - 1)$$

in which I is the tracer infusion rate, E_{tr} the enrichment of the tracer in the infusate, and E_1 and E_4 are the plasma plateau enrichments of [m + 1]methionine and [m + 4]methionine, respectively.

As the rate of appearance equals the rate of disappearance, it follows for Q_m:

$$Q_m = \text{appearance} = D + B + RM = \text{disappearance} = S + TM$$

and for Q_c :

$$Q_c = \text{appearance} = D + B = \text{disappearance} = S + TS$$

in which D is methionine intake via the diet (which is zero during the infusion), B is methionine release from protein breakdown, RM is homocysteine remethylation, S is methionine incorporation in protein synthesis, TM is methionine transmethylation and TS is homocysteine transsulfuration. It follows that:

$$RM = Q_m - Q_c.$$

Transsulfuration rate is calculated from $^{13}CO_2$ excretion in breath air as follows:

$$TS = V^{13}CO_2 * (1/[^{13}C]\text{methionine enrichment in plasma} - 1/[^{13}C]\text{methionine enrichment in tracer infusate}).$$

As methionine is the only precursor of homocysteine, homocysteine disappearance (RM + TS) equals homocysteine appearance (TM); thus:

$$TM = RM + TS.$$

The flux rates for transmethylation, remethylation, and transsulfuration were expressed in μmol/kg fat-free mass/hour. Metabolic homocysteine clearance was calculated with the formula: TS/plasma homocysteine level, and expressed in L/kg fat-free mass/hour.

Statistical methods

Values are expressed as mean ± standard deviation (SD) or as median with range if data were skewed. Differences between mean values before and after treatment within groups were tested with the paired *t* test. For comparisons between groups, the independent *t* test was used. A P value < 0.05 was considered to reflect statistical significance.

RESULTS

All individuals completed the study protocol.

Baseline plasma homocysteine level was significantly elevated in the dialysis patients. Before treatment, remethylation, transmethylation and transsulfuration rates were not significantly different between ESRD and control individuals, whereas metabolic homocysteine clearance was significantly lower in ESRD patients (0.09 ± 0.05 vs. 0.41 ± 0.14 L/kg FFM/hour) ($P < 0.01$).

After treatment, plasma folate concentration increased in all participants. Plasma homocysteine concentration decreased by 48% (95% CI 8 to 88) in the dialysis patients and by 20% (95% CI –1 to +40) in the control group (Table 2). After folic acid treatment, plasma homocysteine concentration was still significantly higher in the dialysis patients compared to the baseline concentration in the control group. In the dialysis patients, there was a significant increase in both the remethylation and transmethylation rate (Table 2). Remethylation increased by 32% (95% CI 5 to 58) and transmethylation by 20% (95% CI 8 to 32). In the control group, remethylation and transmethylation increased non-significantly by 35% (95% CI –14 to +84) and by 11% (95% CI –3 to +25), respectively. In both groups, transsulfuration rate and metabolic homocysteine clearance were not significantly altered by folic acid treatment (Table 2). The remethylation and transmethylation rates after folic acid treatment in the dialysis patients were similar to the baseline levels in the control individuals (both $P = 0.91$) (Figure 2).

Table 2. Plasma homocysteine concentrations, one carbon flux rates, and metabolic homocysteine clearance before and after folic acid treatment in six patients with end-stage renal disease (ESRD) and six healthy controls

	ESRD		Control	
Characteristic	Baseline	After folic acid	Baseline	After folic acid
Plasma homocysteine *µmol/L*	38.7 ± 14.8	20.2 ± 4.1*	8.1 ± 1.4	6.5 ± 1.0‡
Remethylation *µmol/kg fat-free mass/hour*	3.9 ± 1.4	5.1 ± 2.1*	5.0 ± 1.7	6.7 ± 1.1
Transmethylation *µmol/kg fat-free mass/hour*	6.7 ± 1.7	8.0 ± 1.9†	8.1 ± 1.7	9.0 ± 1.8
Transsulfuration *µmol/kg fat-free mass/hour*	2.8 ± 0.7	2.9 ± 1.0	3.2 ± 0.9	2.7 ± 1.1
Metabolic homocysteine clearance *L/kg fat-free mass/hour*	0.09 ± 0.05	0.15 ± 0.05	0.41 ± 0.14	0.43 ± 0.18

Values are mean ± SD. *P < 0.05; †P < 0.01; and ‡P = 0.055 compared to baseline.

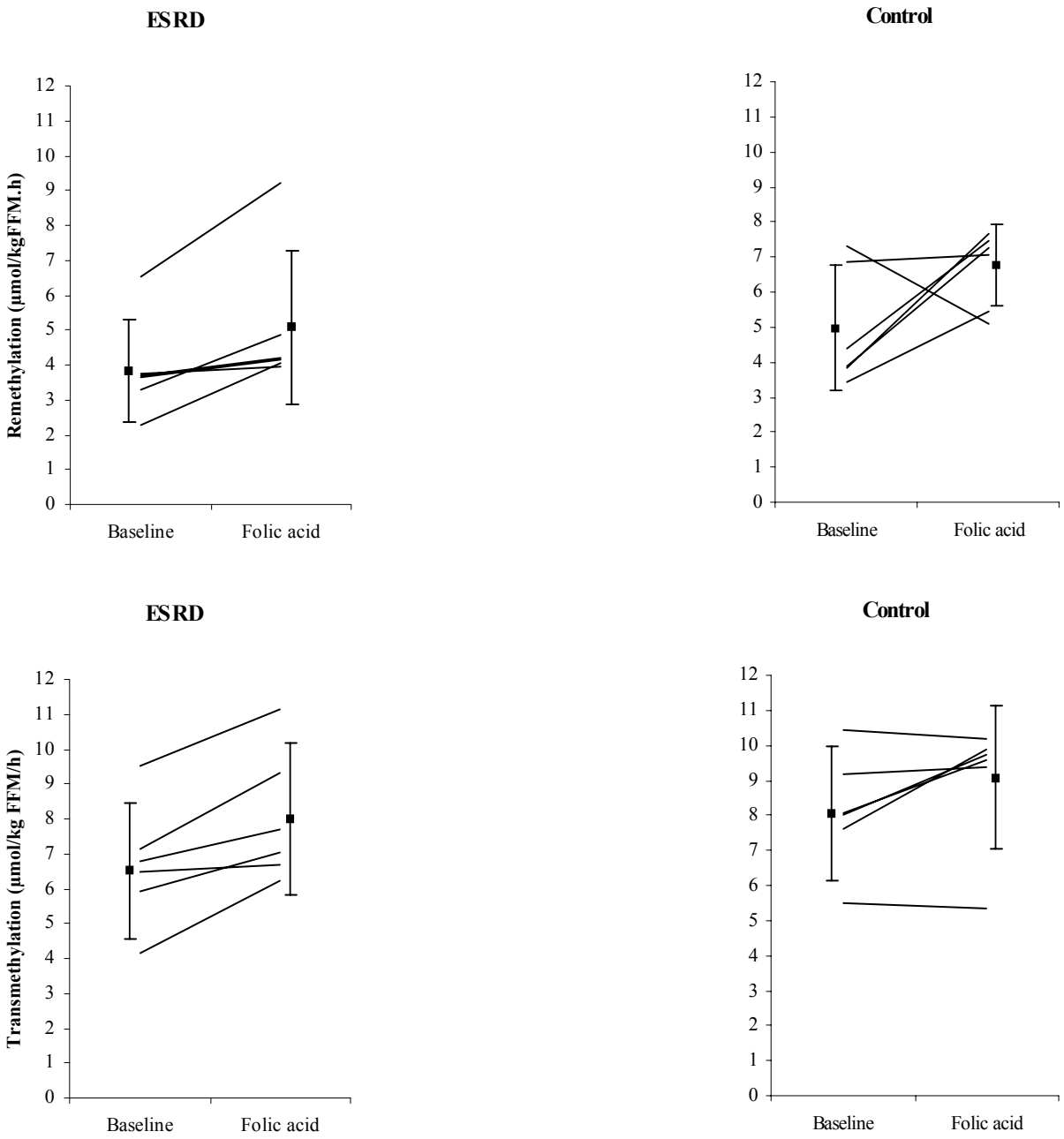

Figure 2. Individual changes in remethylation and transmethylation rate in end-stage renal disease (ESRD) patients and control subjects before and after treatment with folic acid (5 mg/day). Means with SD are depicted as bars.

DISCUSSION

The main findings of this study are that oral treatment with folic acid in ESRD patients (1) lowers, but does not normalize plasma homocysteine concentration, (2) increases whole body homocysteine remethylation and methionine transmethylation rate to normal values, and (3) does not significantly affect transsulfuration rate.

At first glance, one would expect that the net effect of an increase in both remethylation and transmethylation is an unchanged plasma homocysteine level, as the increase in homocysteine removal by remethylation is offset by a similar increase in homocysteine production through transmethylation of methionine. This would leave the decrease in plasma homocysteine unexplained. One possible explanation is that transmethylation is not directly determined in the stable isotope model we used, but calculated as the sum of remethylation and transsulfuration. In steady state, any change in remethylation would be paralleled by the same change in transmethylation if transsulfuration is unchanged, as in our study. One important condition that must be met before this calculation can be applied is the presence of a steady state. From earlier studies, we have established that, after 2 weeks of folic acid treatment in hemodialysis patients, plasma homocysteine levels are stable [19]. Because the participants were prescribed a fixed dietary protein (methionine) intake, steady-state conditions were likely to be present in our study. However, it is possible that in the initial phase of the folic acid treatment, some of the homocysteine remethylates to methionine without increasing transmethylation (possibly by intracellular storage and/or insertion in proteins). After reaching saturation of this process, plasma homocysteine does not decrease further, because increased remethylation is now balanced by an increase in transmethylation.

Another explanation may be that folic acid not only stimulates remethylation, but also improves the metabolic homocysteine clearance. An improved metabolic homocysteine clearance would allow the daily methionine disposal by transsulfuration to take place at a lower plasma homocysteine concentration. In our study, there was an increase in metabolic homocysteine clearance which, however, was not significant, but this could have been due to a type 2 error. Folic acid is not known to have a direct effect on transsulfuration from single enzyme kinetics, but the increase in remethylation could increase levels of methionine and its adenosylated product S-adenosylmethionine. S-adenosylmethionine is a stimulator of β-cystathionine synthase, the rate limiting enzyme of transsulfuration [20].

Our findings may help to explain the cause of hyperhomocysteinemia in ESRD patients. From our pilot study, we have suggested a major role of impaired homocysteine remethylation in the development of hyperhomocysteinemia in chronic renal failure [5]. Although in the present study, baseline remethylation and transmethylation rates in the dialysis patients were not significantly different from controls (possibly a type 2 error), we have demonstrated differences in remethylation rates in a larger study [8]. The present study shows that the remethylation rates in dialysis patients on 5 mg folic acid per day are virtually the same as in comparable healthy subjects not on folic acid, whereas plasma homocysteine

levels are still elevated. Therefore, hyperhomocysteinemia in ESRD patients can only be partially explained by a decreased total body remethylation. An other factor that may play a role is the metabolic homocysteine clearance, which is decreased by ~80% in ESRD [8]. For normalization of plasma homocysteine, restoration of homocysteine clearance (by transsulfuration) seems to be required. Stimulation of the transsulfuration in ESRD patients with vitamin B_6 has been disappointing as it did not result in a significant decrease of fasting plasma homocysteine [21]. Vitamin B_6 seems to have some effect on plasma homocysteine in renal patients only when the transsulfuration pathway is further stressed by methionine loading [22]. Interestingly, we have shown earlier that folic acid is also capable of lowering plasma homocysteine after methionine loading [23] and in the present study, we observed an increase, although not significant, in homocysteine clearance. Together, these observations suggest that folic acid may have a direct or indirect effect on homocysteine transsulfuration; nevertheless, such an effect, even if it exists, is clearly insufficient to normalize plasma homocysteine. Methods that further improve homocysteine clearance by transsulfuration (or by increased extraction during dialysis) are probably necessary to normalize plasma homocysteine in ESRD patients.

Folic acid treatment in ESRD patients was paralleled by an increase in methionine transmethylation in our study. Transmethylation of methionine provides methyl groups to the majority of methyl group acceptors in the body, such as DNA. Recently, Ingrosso et al. [24] have shown that DNA in leukocytes of ESRD patients is hypomethylated and that this could be restored by folate treatment. The authors suggested that this effect is mediated by an improved transmethylation. The present study shows that folic acid is indeed capable of increasing transmethylation flux in dialysis patients. We conclude that folic acid treatment in ESRD patients results in an increase of homocysteine remethylation and methionine transmethylation rate to normal levels and an unaltered transsulfuration rate. These changes in fluxes are accompanied by a lower, but still elevated plasma homocysteine concentration. Studies aimed at amelioration of hyperhomocysteinemia in ESRD by stimulation of homocysteine clearance (by transsulfuration) are now warranted.

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Chapter 5

Folic acid treatment increases homocysteine remethylation and methionine transmethylation in healthy subjects

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ABSTRACT

Folic acid treatment decreases plasma total homocysteine concentration in healthy subjects, but the effects on homocysteine metabolism are unknown. In the present study, we investigated the effect of 3 weeks of oral treatment with 5 mg folic acid on one-carbon flux rates in 12 healthy subjects, using in vivo stable isotope methods. In addition, we determined the effect of folic acid on blood concentrations of amino acids which may have regulatory roles in homocysteine metabolism, i.e. homocysteine, AdoMet (S-adenosylmethionine), AdoHcy (S-adenosylhomocysteine), serine and glycine. Primed, continuous infusions with [$^{2}H_{3}$-methyl-1-^{13}C]methionine were used to determine flux rates of methionine transmethylation, homocysteine remethylation and homocysteine transsulphuration. Metabolic homocysteine clearance was defined as the ratio of transsulphuration and plasma homocysteine level. Folic acid treatment increased homocysteine remethylation rate by 59% [95% CI (confidence interval) 13 to 97, P = 0.02] and methionine transmethylation rate by 20% (95% CI 3 to 41, P = 0.03). Plasma total homocysteine concentration (–18%; 95% CI –28 to –9, P < 0.01) and serine/glycine ratio (–20%; 95% CI –63 to –6, P < 0.01) decreased significantly, and AdoMet/AdoHcy ratio (11%; 95% CI 1 to 20, P = 0.02) increased significantly. Changes in one-carbon flux rates did not correlate significantly with changes in plasma concentration of these amino acids. In conclusion, folic acid treatment lowered plasma homocysteine concentration and increased whole-body remethylation and transmethylation flux in healthy subjects.

INTRODUCTION

Homocysteinaemia is a continuous independent risk factor for cardiovascular disease [1]. Blood concentrations of folate and pyridoxine (vitamin B_6) and of cobalamin (vitamin B_{12}) are determinants of homocysteinaemia, even in the normal range of the plasma homocysteine concentrations [2,3]. In turn, the nutritional status for folate and pyridoxine (vitamin B_6) is inversely associated with the risk of coronary heart disease [4,5]

Homocysteine metabolism is complex (Figure 1), with a central role for folate, a compound name for a range of biochemical forms of this B-vitamin. Conceivably, the steady-state plasma homocysteine concentration is only partly determined by the rate of homocysteine remethylation. Rather, remethylation, transmethylation and transsulphuration act in concert on homocysteine homeostasis. Remethylation, transmethylation and transsulphuration have been thought to be regulated by the concentrations of the B vitamins involved and the (genetically determined) activity of specific enzymes, but also by the concentration of intermediate metabolites in homocysteine metabolism. For example, single enzyme kinetic studies have provided evidence that AdoMet (S-adenosylmethionine) stimulates homocysteine transsulphuration by activating cystathionine β-synthase and inhibits methionine remethylation by inhibiting methylenetetrahydrofolate reductase [7]. In contrast, AdoHcy (S-adenosylhomocysteine) stimulates both homocysteine transsulphuration and remethylation by activating cystathionine ß-synthase and methionine synthase [7]. The ratio of AdoMet to AdoHcy determines the activity of the methyltransferases involved in methionine transmethylation [7].

Folic acid (pteroylglutamic acid) has been the cornerstone of most homocysteine-lowering regimens [8], and has been shown to lower plasma homocysteine even in healthy, folate-replete, normohomocysteinaemic subjects [9]. However, its homocysteine-lowering mechanism is not entirely clear. By conversion into 5-methyltetrahydrofolate, folic acid is thought to stimulate remethylation. However, it is unknown how exactly the homocysteine-lowering effect of folic acid is quantitatively related to folic-acid-induced changes in whole-body remethylation. It is arguable that this may not be a simple linear relationship, because plasma total homocysteine concentrations are an indirect reflection of actual homocysteine concentrations in a wide array of different intracellular compartments. In addition, increased intracellular AdoMet concentrations, in response to folic-acid-stimulated remethylation, may stimulate homocysteine transsulphuration and inhibit homocysteine remethylation [10].

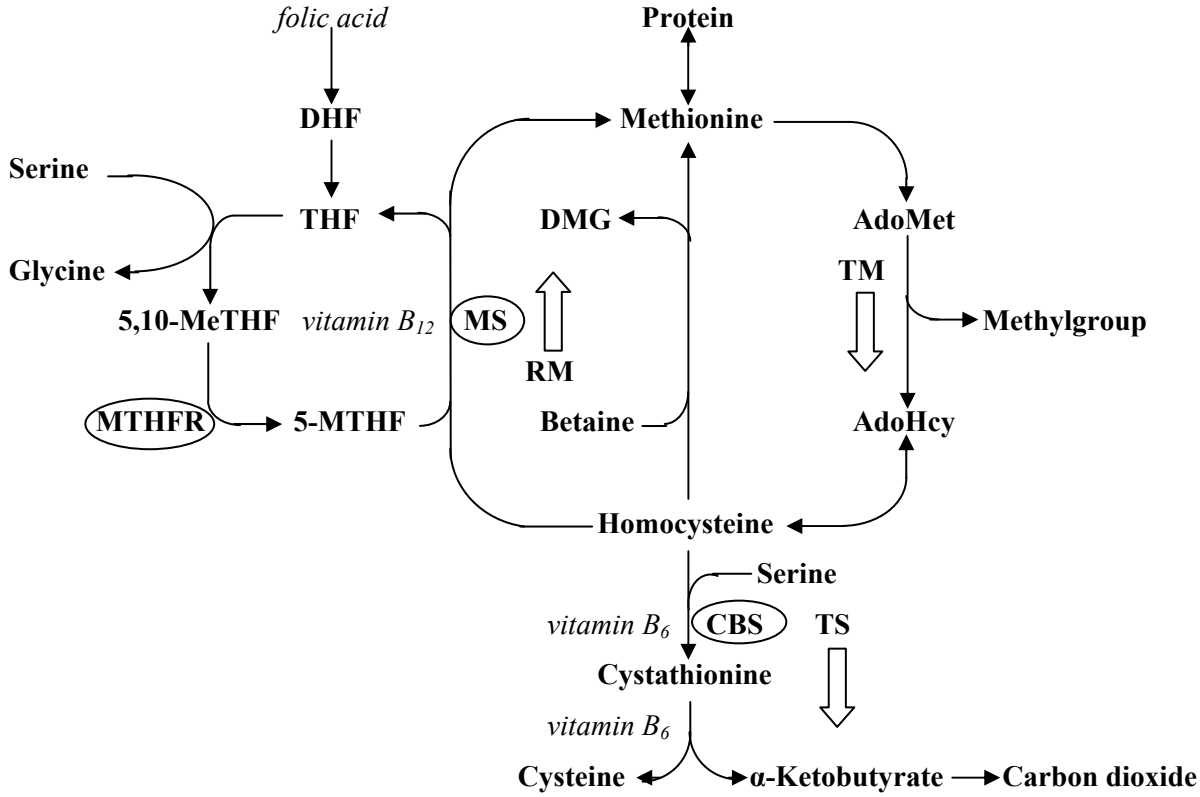

Figure. 1. An overview of homocysteine metabolism. A concise overview of homocysteine metabolism, with open arrows indicating metabolic fluxes, ovals indicating enzymes and italics indicating vitamins. Homocysteine is the transmethylation (TM) product of the essential sulphur-containing amino acid methionine, with AdoMet and AdoHcy as intermediates. Homocysteine can be either remethylated to methionine or degraded by transsulphuration (TS). In the folate-dependent remethylation reaction, which is catalysed by methionine synthase (MS) and uses vitamin B_{12} as a cofactor, 5-methyltetrahydrofolate (5-MTHF) donates a methyl group to homocysteine. Subsequently, tetrahydrofolate (THF) receives a methylene group from the serine/glycine couple, a reaction that uses vitamin B_6 as a co-factor. Tetrahydrofolate can also be generated by reduction of supplemented synthetic folic acid to dihydrofolate (DHF) and subsequently to THF. Next, 5,10-methylenetetrahydrofolate (5,10-MeTHF) is reduced to 5-MTHF, requiring the enzyme methylenetetrahydrofolate reductase (MTHFR). Another folate-independent remethylation reaction, which is quantitatively unimportant in humans in physiological conditions [6], uses betaine as a methyl group donor, generating dimethylglycine (DMG). In the irreversible, catabolic (transsulphuration) pathway, the rate-limiting reaction is catalysed by cystathionine β-synthase (CBS) and requires the active form of vitamin B_6 as a cofactor.

The aim of the present study was to quantify folic-acid-induced changes in whole-body one-carbon flux rates in healthy subjects, using stable isotope methods [11,12]. In addition, we determined the effect of folic acid on plasma concentrations of homocysteine, AdoMet, AdoHcy, serine and glycine, which may have regulatory roles in homocysteine metabolism.

METHODS

Subjects

Twelve healthy adults (five active smokers) were studied. Baseline characteristics are given in Table 1. Subjects who used vitamin supplements or drank more than two units of alcohol a day were not included. None of the participants used any medication.

Table 1. Baseline characteristics

Sex (male/female)	6/6
Age (years)	29 (19–64)
Body mass index (kg/m^2)	22 (19–30)
Body weight (kg)	65 (57–108)
Fat-free mass (kg)	52 (40–90)
Serum creatinine (μmol/l)	89 (62–105)
Genotype	
5,10-Methylenetetrahydrofolate reductase	8 CC, 4 CT and 0 TT
Cystathionine β-synthase	9 -/-, 3 -/+ and 0 +/+
Methionine synthase	10 AA, 1 AG and 1 GG

Values of continuous variables are presented as medians (range). CC denotes wild-type; CT, heterozygous for C677T; TT, homozygous for C677T; -/-, wild-type; -/+, heterozygous for 844ins68; +/+, homozygous for 844ins68; AA, wild-type; AG, heterozygous for A2756G; GG, homozygous for A2756G.

The study protocol was approved by the Ethics Committee of the VU University Medical Center, and all participants gave their written informed consent.

Experimental protocol

Subjects were instructed to use oral supplements of folic acid for 21 days (one tablet containing 5mg of folic acid daily at 18:00 hours). Stable isotope infusion was performed at baseline and after 3 weeks of folic acid treatment, where the last folic acid tablet was taken the day before the second measurement. The study protocol concerning the stable isotope infusion has been described in detail elsewhere [11]. Briefly, the participants were kept on a stable protein diet of 1.0 g · kg^{-1} of body weight · day^{-1} for 3 days prior to the experiment. All subjects remained fasting and recumbent during the test. At 08:00 hours, two intravenous catheters were placed in a dorsal hand vein, one for infusion of substances and one, in the contralateral hand, for sampling. Arterialized blood samples were drawn from the dorsal hand vein after the hand was inserted in a heated box [13]. Blood was collected in heparinized glass tubes, immediately placed on ice and centrifuged for 10 min at 1000 *g* at –4ºC within 15 min. Plasma was stored at –30ºC until analysed. Samples of end-tidal expired breath air were collected in a 15 ml Venoject® tube by instructing the subjects to exhale through a straw.

During the last 3 s of expiration, the straw was withdrawn from the tube, which was immediately closed by the investigator. After baseline samples were taken, a priming bolus of 5.9 μmol $NaH^{13}CO_3$ (99% [^{13}C]; ARC Laboratories, Apeldoorn, The Netherlands) was administered, followed by a primed (3.5 μmol/kg of body weight) constant infusion of L-[$^{2}H_3$-methyl-1-^{13}C]methionine (95% doubly labeled; 99% [1-^{13}C]; 99% [$^{2}H_1$]; Isotec, Miamisburg, OH, U.S.A.) at a rate of 2.2 μmol kg^{-1} of body weight hr^{-1} for 5 h. Plateau-enrichment concentrations were calculated as the mean of the final five 20-min interval samples of the infusion period. Body weight was measured on a balance scale (accuracy 50 g), and four skinfolds were measured using a calliper (Holtain; accuracy 0.1 mm). FFM (fat-free mass) was calculated from skinfold measurements according to Durnin and Womersley [14].

Laboratory analyses

Plasma total (free plus protein bound) homocysteine was measured with the use of a microparticle enzyme immunoassay method based on fluorescence polarization (IMX analyser; Abbott, Chicago, IL, U.S.A.). Intra- and inter-assay CVs (coefficients of variation) were 2.1 and 5.1% respectively. Serine (inter-assay CV, 3%) and glycine (inter-assay CV, 3%) analyses were performed by HPLC after precolumn derivatization with orthophtalaldehyde. AdoMet and AdoHcy were measured in plasma and whole blood using stable-isotope dilution tandem MS [15]. The intra-assay CVs for AdoMet and AdoHcy were 4.2 and 4.0% respectively, and the inter-assay CVs for AdoMet and AdoHcy were 7.6 and 5.9% respectively. Serum creatinine was measured by means of modified Jaffé method. Serum folate (intra- and inter-assay CVs, 4 and 5% respectively) and vitamin B_{12} (intra- and inter-assay CVs, 4 and 5% respectively) were measured by radioassay (ICN, Costa Mesa, CA, U.S.A.), and serum vitamin B_6 (inter-assay CV 7%) with the use of fluorescence HPLC [16]. The methionine concentration in the infusate was measured with an amino acid analyser equipped with a high-pressure analytical column packed with Ultrapac 8 resin (Pharmacia Biotech, Cambridge, U.K.). Isotopic enrichments of methionine in plasma was measured in the acetyl-3,5-bis(trifluoromethyl)benzyl derivative with the use of GC-MS, as previously described [17]. Enrichments, expressed in MPE (mole percent enrichment), were calculated on the basis of the abundance relative of the (m + 0), (m + 1) and (m + 4) methionine species [18], and calibration curves obtained from weighed amounts of tracer (*m* + 1 and *m* + 4) and tracee methionine were used to correct for minor instrument variation [17]. The [^{13}C]-enrichment of CO_2 in breath samples was measured on a dual-inlet isotope ratio mass

spectrometer (VG OPTIMA; Fisons Instruments, Middlewich, Cheshire, U.K.) and expressed in APE (atom percent excess).

Gene polymorphisms

The polymorphisms of 5,10-methylenetetrahydrofolate reductase (C677T transition), cystathionine β-synthase (844ins68 variant) and methionine synthase (A2756G transition) were assessed in DNA obtained from the buffy coat of EDTA-collected blood as described by Tsai et al. [19].

Calculations

L-[$^{2}H_3$-methyl-1-^{13}C]methionine was used as a tracer, according to the method described by Storch et al. [18]. This stable isotope has a molecular weight of m + 4 relative to natural methionine (m). The $^{2}H_3$-methyl label is removed from methionine during transmethylation and thus [$^{2}H_3$-methyl-1-^{13}C]methionine is converted into [1-^{13}C]homocysteine. Remethylation will result in the generation of m + 1 methionine, because the [^{13}C] atom of the carboxyl moiety of homocysteine remains intact. In contrast, during transsulphuration, the carboxyl moiety of [1-^{13}C]homocysteine loses its [^{13}C] atom. When α-ketobutyrate is oxidised in the Krebs cycle, the label ultimately appears as $^{13}CO_2$ in breath air. The m + 4 methionine tracer is diluted by methionine entering the pool via the diet, from homocysteine folate-dependent remethylation and by free methionine entering from protein breakdown in the tissues. In the steady state, the rate of appearance of methionine from these sources equals the rate of disappearance (i.e. protein synthesis and transmethylation). In this model, it is assumed that the intracellular and intravascular compartments are in rapid and complete isotopic equilibrium.

From the plasma enrichments of methionine, the whole-body methionine-methyl rate of appearance and disappearance (Q_m) and methionine-carboxyl rate of appearance and disappearance (Q_c) are calculated as follows:

$$Q_m = I \times (E_{tr} / E_4 - 1)$$
$$Q_c = I \times [E_{tr} / (E_1 + E_4) - 1]$$

where I is the tracer infusion rate, E_{tr} the enrichment of the tracer in the infusate, and E_1 and E_4 are the plasma plateau enrichments of m + 1 and m + 4 methionine respectively.

As the rate of appearance equals the rate of disappearance, it follows for Q_m:

$$Q_m = \text{appearance} = D + B + RM = \text{disappearance} = S + TM$$

and for Q_c:

$$Q_c = \text{appearance} = D + B = \text{disappearance} = S + TS$$

where D is methionine intake via the diet (which is zero during the infusion protocol), B is methionine release from protein breakdown, RM is homocysteine remethylation, S is methionine incorporation in protein synthesis, TM is methionine transmethylation, and TS is homocysteine transsulphuration. It follows that:

$$RM = Q_m - Q_c.$$

The transsulphuration rate is calculated from $^{13}CO_2$ excretion in breath air as follows:

$$TS = V^{13}_{CO2} \times (1/[^{13}C]\text{methionine enrichment in plasma} - 1/[^{13}C]\text{methionine enrichment in tracer infusate}).$$

Note that we made the assumption that, in healthy subjects, the transamination pathway is negligible [20]. However, if the transamination pathway is significant, $^{13}CO_2$ from the [^{13}C]carboxyl-labelled tracer would arise via AdoMet-independent pathways, leading to overestimation of transsulphuration and transmethylation in our model. Nevertheless, the estimate of total methionine oxidation would not be affected.

As methionine is the only precursor of homocysteine, homocysteine disappearance (RM + TS) equals homocysteine appearance (TM), thus TM = RM + TS.

The flux rates for remethylation, transmethylation and transsulphuration were expressed as μmol · h^{-1} · kg^{-1} body weight and μmol · h^{-1} · kg^{-1} of FFM. Because the metabolic homocysteine clearance by transsulphuration reflects homocysteine disposal capacity in the steady state [21], we also calculated homocysteine clearance by transsulphuration with the formula: [TS (as expressed per kg of FFM) x FFM]/total plasma homocysteine.

Statistical methods

Data were analysed using SPSS version 11.5.0 (SPSS Inc, Chicago, IL, U.S.A.). CIs (confidence intervals) were calculated by CIA version 2.0.0 (Medical Statistics Computing, Southampton, U.K.). The plateau isotopic-enrichment levels were analysed by visual

inspection and ANOVA. Values are expressed as medians (range). Differences between medians are expressed as relative changes from baseline in percentages with 95% CIs. Differences between values before and after treatment were tested with the Wilcoxon signed-ranks test. Differences between subgroups were tested with the Mann-Whitney test. Pearson's test was used for correlation analyses. For calculation of correlations with flux rates, the flux rates were expressed per kg of FFM. A P value < 0.05 was considered to reflect statistical significance.

RESULTS

All 12 subjects completed the study protocol. Plateau plasma methionine and breath air $^{13}CO_2$ enrichments were obtained in all individuals. Plateau was reached after 220 min. Conditions for MS measurements were similar before and after folic acid treatment (isotopic enrichment ranged between 1–2 MPE for [1-^{13}C]methionine and between 5 to 10 MPE for [$^{2}H_3$-methyl-1-^{13}C]methionine, and breath air $^{13}CO_2$ was > 0.002 APE in each subject).

The effects of treatment with folic acid on blood concentrations of vitamins and amino acids are shown in Table 2. In summary, all subjects showed an increase in the serum folate concentration (284%; 95% CI 136 to 629). Compared to baseline, plasma total homocysteine decreased by 18% (95% CI –28 to –9%). The ratio of AdoMet and AdoHcy in plasma increased significantly by 11% (95% CI 1 to 20), which was not the case in whole blood.

Table 2. Effects of folic acid treatment (5 mg/day) on vitamins and amino acids involved in methionine and homocysteine metabolism in 12 healthy subjects

	At baseline	After folic acid	*P* value
Serum folate (nmol/l)	14.6 (7.3–18.7)	42.0 (14.1–180.0)	< 0.01
Serum vitamin B_6 (nmol/l)	29 (8–58)	24 (12–55)	0.92
Serum vitamin B_{12} (pmol/l)	223 (175–332)	215 (168–424)	0.48
Plasma total homocysteine (μmol/l)	7.4 (5.6–10.6)	6.4 (4.5–8.2)	< 0.01
Plasma serine (μmol/l)	90 (75–112)	88 (71–120)	0.06
Plasma glycine (μmol/l)	184 (52–241)	199 (74–279)	0.03
Plasma serine/lycine ratio	0.51 (0.37–1.73)	0.47 (0.32–1.20)	< 0.01
S-adenosylmethionine			
In plasma (nmol/l)	88 (61–112)	89 (73–120)	0.70
In whole blood (nmol/l)	1580 (1095–1865)	1483 (984–1910)	0.25
S-adenosylhomocysteine			
In plasma (nmol/l)	12 (9–17)	11 (8–18)	0.28
In whole blood (nmol/l)	62 (47–88)	53 (39–97)	0.29
S-adenosylmethionine/ S-adenosylhomocysteine ratio			
In plasma	6.8 (5.7–10.4)	7.6 (5.9–10.4)	0.02
In whole blood	25.1 (13.9–39.7)	25.0 (18.7–38.1)	0.42

Values are presented as medians (range). P values were determined using the Wilcoxon signed-ranks test.

Changes in plasma and whole-blood concentrations of AdoMet and AdoHcy separately did not reach significance. In plasma, glycine increased significantly by 10% (95% CI 2 to 22) and serine showed a decrease that did not reach the level of significance (–7%; 95% CI –12 to +1). The serine/glycine ratio decreased significantly by 20% (95% CI –63 to –6).

After folic acid treatment, there was a significant increase in (for FFM corrected) remethylation rate (59%; 95% CI 13 to 97) and transmethylation rate (20%; 95% CI 3 to 41). Changes in transsulphuration rate (–9%; 95% CI –27 to +11) and metabolic homocysteine clearance by transsulphuration (8%; 95% CI –18 to +35) were not significant (Table 3). There were no significant differences in changes in flux rates and homocysteine clearance between men and women, and smokers and non-smokers (results not shown).

Table 3. Effects of folic acid treatment (5 mg/day) on one-carbon flux rates of methionine and homocysteine metabolism in 12 healthy subjects in the postabsorptive state

	At baseline	After folic acid	P-value
Remethylation			
Expressed in body weight (μmol · kg^{-1} BW · h^{-1})	4.0 (2.5–7.0)	6.3 (3.8–10.5)	0.02
Expressed in fat-free mass (μmol · kg^{-1} FFM · h^{-1})	4.6 (3.4–10.1)	7.6 (4.7–12.5)	0.02
Transmethylation			
Expressed in body weight (μmol · kg^{-1} BW · h^{-1})	7.1 (4.0–9.8)	8.3 (3.9–13.7)	0.02
Expressed in fat-free mass (μmol · kg^{-1} FFM · h^{-1})	9.3 (5.5–14.2)	10.1 (5.3–16.3)	0.03
Transsulphuration			
Expressed in body weight (μmol · kg^{-1} BW · h^{-1})	2.9 (1.5–4.6)	2.3 (1.7–5.1)	0.27
Expressed in fat-free mass (μmol · kg^{-1} FFM · h^{-1})	4.0 (2.1–5.5)	3.1 (2.1–6.2)	0.24
Homocysteine clearance by transsulfuration			
Expressed in body weight (l · kg^{-1} of BW · h^{-1})	0.41 (0.20–0.75)	0.35 (0.25–0.98)	0.48
Expressed in fat-free mass (l · kg^{-1} of FFM · h^{-1})	0.51 (0.23–0.91)	0.49 (0.30–1.18)	0.58
Methionine methyl flux			
Expressed in body weight (μmol · kg^{-1} BW · h^{-1})	22.1 (17.0–34.1)	21.8 (18.2–29.9)	0.64
Expressed in fat-free mass (μmol · kg^{-1} FFM · h^{-1})	27.2 (23.3–49.2)	28.5 (22.4–35.7)	0.64
Methionine carboxyl flux			
Expressed in body weight (μmol · kg^{-1} BW · h^{-1})	18.4 (14.0–27.1)	14.9 (12.7–22.5)	< 0.01
Expressed in fat-free mass (μmol · kg^{-1} FFM · h^{-1})	22.9 (16.8–39.1)	20.8 (15.3–30.6)	< 0.01

Values are presented as medians (range). BW denotes body weight; FFM, fat-free mass. P values were determined using the Wilcoxon signed-ranks test.

Figure 2 shows the effect of folic acid supplementation on remethylation and transmethylation rates of subjects. The two subjects with the highest baseline remethylation rates showed decreases in both remethylation rate as well as transmethylation rate, yet exhibited a decrease in plasma total homocysteine. These two subjects were homozygous wild-type for the C677T transition of the methylenetetrahydrofolate reductase gene, the 844ins68 variant of the cystathionine β-synthase gene, as well as the A2756G transition of the methionine synthase gene, and had baseline concentrations of folate and increases of folate concentrations that were comparable with the concentrations in the other ten subjects (results not shown).

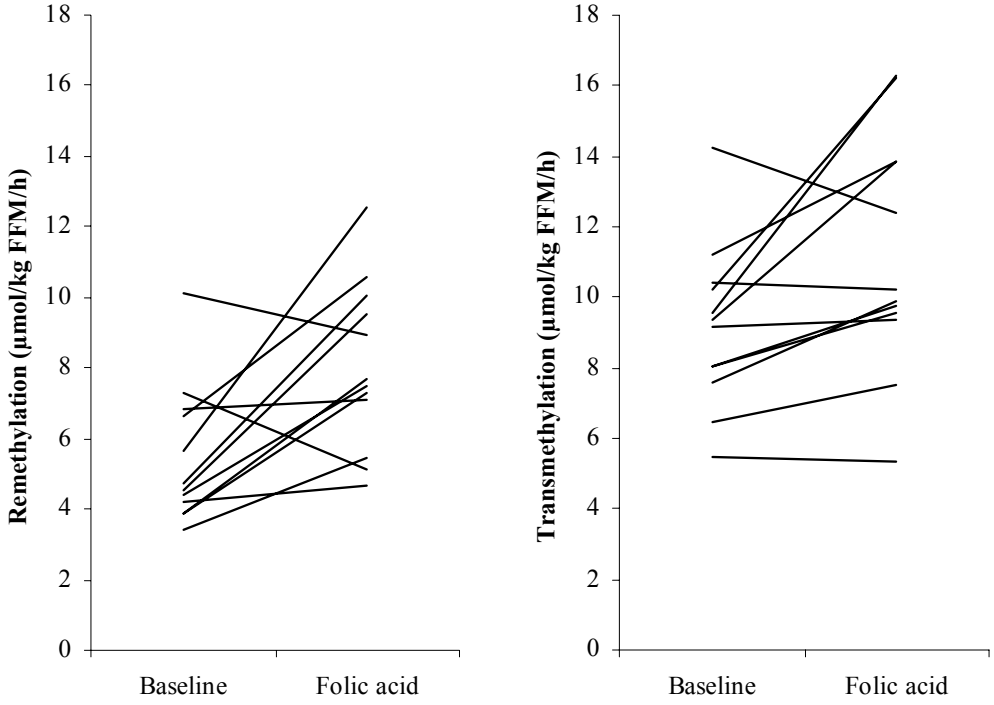

Figure 2. Individual changes in remethylation and transmethylation after treatment with folic acid (5 mg/day).

Changes in remethylation rate did not correlate significantly with changes in plasma total homocysteine concentration (r = –0.38; 95% CI –0.74 to +0.14, P = 0.22). No significant correlations could be demonstrated between changes in any of the one-carbon fluxes and changes in blood concentrations of total homocysteine, AdoMet, AdoHcy, serine, glycine, or B vitamins (results not shown).

DISCUSSION

The results of the present study show that, in healthy subjects, treatment with folic acid increased rates of whole-body homocysteine remethylation and methionine transmethylation, and decreased plasma total homocysteine concentration. Folic acid supplementation did not significantly change homocysteine transsulphuration or metabolic homocysteine clearance. In addition, the present study shows that folic acid increased the plasma AdoMet/AdoHcy ratio and decreased the serine/glycine ratio.

The 18% decrease in plasma homocysteine concentration after folic acid treatment was comparable with the 16% decrease which was found in the meta-analysis of the

Homocysteine Lowering Trialists' Collaboration in the subgroup of patients with baseline plasma homocysteine concentrations < 8.9 μmol/l [8].

We did not find a significant relationship between changes of plasma homocysteine concentration and remethylation rate. Moreover, two subjects demonstrated a decrease in the plasma homocysteine concentration as well as in the remethylation rate. In one subject, this might be explained by the simultaneous increase of homocysteine clearance, which might have accounted for the decrease in plasma homocysteine concentration. In the other subject, a statistical, rather than a biological, explanation for the combined decrease in remethylation rate and plasma homocysteine concentration seems more likely (regression to the mean). In this regard, it should be stressed that our study was not designed (and thus not powered) to examine relationships between the one-carbon fluxes and plasma concentrations of the various compounds, but rather to examine their mean changes after folic acid supplementation.

It is possible that in the initial phase of folic acid treatment, an increase in homocysteine remethylation rate lowered the plasma homocysteine concentration (for example by intracellular storage and/or insertion of methionine in proteins). A secondary increase in methionine transmethylation rate could balance the increased remethylation rate at a lower plasma homocysteine concentration. The transsulphuration rate was unchanged after folic acid treatment. In weight-maintaining adults, the transsulphuration rate reflects oxidation of methionine from dietary intake, because methionine is the only precursor of homocysteine, and transsulphuration is the only way of homocysteine disposal (methionine transamination is negligible in normal subjects [20]). In the design of our present study, protein intake was kept constant, and it is thus not surprising that transsulphuration rate was not affected by folic acid treatment. However, it has to be kept in mind that our results reflect the status of methionine and homocysteine metabolism reached after 3 weeks of folic acid treatment, which, inherent to the stable isotope model, do not necessarily reflect previous (possibly transient) changes in flux rates and metabolite concentrations.

Dose-response effects of supplemented folic acid on folate and homocysteine concentrations in blood have shown to flatten at intakes of 400 μg/day [22,23], a dose that could be reached with food fortification [24]. However, the quantitative effects of folic acid supplementation on methyl flux rates are unknown. Therefore, it is not clear whether a similar threshold exists for changes in methyl flux rates. Thus we applied a high dose of folic acid with the intention to stimulate remethylation flux maximally. It is not known whether a lower folic acid dose (e.g. 400 μg) induces similar changes in one-carbon fluxes as in our present

study. Therefore studies on homocysteine metabolism at the internationally recommended daily folic acid intake of 200 µg [25,26] to 400 µg [27] are warranted.

In our present study, methionine transmethylation was increased by folic acid supplementation. An increase in transmethylation may play a crucial role in any beneficial effect of folic acid intervention. There are indications that hypomethylation, as reflected by DNA methylation status, is important in the pathogenesis of atherosclerosis [28,29]. In addition, patients with manifest vascular disease had low plasma AdoMet/AdoHcy ratios in conjunction with DNA hypomethylation [30]. Hypomethylation can be reversed by folic acid treatment, as was demonstrated in end-stage renal disease patients by Ingrosso et al. [31]. However, our study suggests that changes in transmethylation rate may not accurately be reflected by changes in plasma homocysteine concentration in healthy subjects. Thus the degree of homocysteine concentration reduction may not be a good predictor of clinical benefit of folic-acid-based interventions [32].

Folic acid therapy lowered the serine/glycine ratio in the present study. Serine is, by conversion into glycine, a major donor of one-carbon units used in the folate-dependent remethylation of homocysteine to methionine [33]. The observed decrease in the serine/glycine ratio is consistent with the use of serine as a one-carbon donor [6]. Glycine itself is needed for several biosynthetic pathways, including creatine, porphyrins, purines, bile acids and glutathione [34]. It can be hypothesised that the folic-acid-induced changes in the serine/glycine ratio might have affected these biosynthetic pathways, but the exact implications of these findings are unknown.

Taken together, our data show that oral folic acid administration lowers plasma homocysteine concentrations and increases whole-body remethylation and transmethylation fluxes.

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Chapter 6

Impaired renal function is associated with markers of endothelial dysfunction and increased inflammatory activity

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ABSTRACT

Background. Patients with end-stage renal disease (ESRD) as well as those with mild renal insufficiency are at increased risk for the development of cardiovascular disease, which cannot be attributed entirely to traditional risk factors. Endothelial dysfunction and chronic inflammatory activity, two important phenomena in atherogenesis, can be found in ESRD. At present, it is unclear whether endothelial dysfunction and chronic inflammatory activity are related to renal function in the pre-dialysis stage.

Methods. In a cross-sectional, single-centre study, four groups of twenty subjects with renal function ranging from a normal, calculated creatinine clearance (> 90 ml/min) to a pre-dialysis situation (< 31 ml/min) were investigated. We measured markers of endothelial function [von Willebrand factor (vWf), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular-cell adhesion molecule-1 (sVCAM-1), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and E-selectin (ES)], and markers of inflammatory activity [secretory phospholipase A_2 ($sPLA_2$) and C-reactive protein (CRP)]. Using these markers, composite endothelial function and inflammatory activity scores were constructed.

Results. Creatinine clearance correlated with the endothelial function score ($r = –0.43$, $P < 0.001$), the inflammatory activity score ($r = –0.53$, $P < 0.05$), vWf ($r = –0.54$, $P < 0.001$), sVCAM-1 ($r = –0.50$, $P < 0.001$), $sPLA_2$ ($r = –0.28$, $P < 0.05$), homocysteine ($r = –0.61$, $P < 0.001$), age ($r = –0.54$, $P < 0.001$) and blood pressure ($r = –0.44$, $P < 0.001$). In multivariate analyses, creatinine clearance was an independent determinant of the endothelial function score ($\beta = –0.34$, $P < 0.01$), plasma vWf ($\beta = –0.37$, $P < 0.05$) and sICAM-1 ($\beta = –0.33$, $P < 0.05$). The relationship of creatinine clearance with sVCAM-1 and endothelial function score was not significant when plasma homocysteine was added to the model. Creatinine clearance was also a determinant of the inflammatory activity score ($\beta = –0.31$, $P = 0.025$) and $sPLA_2$ ($\beta = –0.32$, $P < 0.05$), although this was no longer significant after correction for systolic blood pressure.

Conclusions. Renal dysfunction is associated with markers of endothelial dysfunction and inflammatory activity. Plasma homocysteine may be an intermediate factor in the relationship between endothelial dysfunction and renal function, while blood pressure may modulate the association between inflammatory activity and renal function.

INTRODUCTION

Patients with end-stage renal disease (ESRD) are at high risk of developing cardiovascular disease (CVD), which limits their life expectancy considerably [1]. Prospective studies in patients with milder degrees of renal impairment have also shown that a reduced glomerular filtration rate (defined as elevated serum creatinine level or reduced creatinine clearance) is associated with an increased (cardiovascular) mortality and morbidity [2,3]. This excess risk cannot be attributed totally to established cardiovascular risk factors. Therefore, the nature of the link between renal function and CVD remains to be explained.

On the one hand, renal insufficiency may be part of a generalized (subclinical) atherothrombotic process. On the other, it is conceivable that a reduction in renal function creates a atherogenic milieu, e.g. by the retention of vasotoxic substances or by metabolic changes that may lead to increased oxidative stress or an enhanced (low-grade) inflammatory state. Such changes may cause endothelial dysfunction, which is an important first step in atherogenesis [4].

Endothelial dysfunction, characterized by increased plasma concentrations of endothelium-derived proteins or reduced endothelium-dependent vasodilatation, has been demonstrated in ESRD patients [5]. However, it is unknown whether this is the result of the lost kidney function or merely reflects the high prevalence of established vascular disease in ESRD patients. In patients with pre-dialysis renal failure, endothelium-dependent vasodilatation is also reduced [6]. In addition, some biochemical markers of endothelial dysfunction, such as von Willebrand factor (vWf) and soluble vascular cell adhesion molecule-1 (sVCAM-1), may be increased [6]. It remains unclear, however, whether renal function per se is related to this endothelial dysfunction.

Plasma levels of pro-inflammatory response markers, such as C-reactive protein (CRP), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), are also increased in pre-dialysis renal patients [6]. However, the relationship between inflammatory activity and creatinine clearance or endothelial function is not well defined in this population. We hypothesised that renal insufficiency per se contributes to endothelial dysfunction and inflammatory activity, and as such may be causally related to CVD.

In view of these considerations, we investigated, in individuals with a wide range of creatinine clearance, whether impaired renal function was associated with changes in plasma concentrations of markers of endothelial dysfunction and/or of inflammatory activity.

SUBJECTS AND METHODS

Subjects

The protocol was approved by the Ethics Committee of the VU University Medical Centre, and all patients gave their informed consent. Individuals were recruited from the outpatient clinics of the Departments of Nephrology and Internal Medicine of the VU University Medical Centre. Exclusion criteria were age younger than 18 years or older than 75 years, haemodialysis, peritoneal dialysis, renal transplantation, diabetes mellitus (ADA criteria), inflammatory diseases, WHO performance > 2, other severe disease (malignancy or liver disease) or use of folic acid, immunosuppressive drugs, penicillamine or anticonvulsant drugs.

Study design

Eighty subjects were included in this cross-sectional, single-centre study. Consecutive patients were included until four groups of 20 subjects were formed, with a creatinine clearance (calculated with the formula of Cockcroft and Gault as a surrogate marker for the glomerular filtration rate) of ≥ 91 ml/min, 61 to 90 ml/min, 31 to 60 ml/min and < 31 ml/min. This was performed to ensure a balanced representation throughout the whole spectrum of renal function. After correcting the creatinine clearance for body surface area, the group was divided into quartiles. The first quartile (with the highest creatinine clearance) served as the control group. In 65 (81%) of the 80 individuals, a renal disease was diagnosed: hypertensive nephrosclerosis in 20, polycystic kidney disease in 15, primary glomerular disease in 14, urolithiasis in four, vasculitis in four, chronic pyelonephritis in two, Alport syndrome in two and other renal diseases in four. Most of the 15 (19%) patients without renal disease were evaluated for chronic fatigue or treated for thyroidal illness. Of these 15 patients without renal disease, 10 were included in the control group and five in the group with a creatinine clearance between 61 and 90 ml/min. For each patient, data were collected with regard to age, cardiovascular medication (antihypertensive medication, platelet aggregation inhibitors and lipid-lowering drugs), smoking status (having smoked in the past year), presence of CVD (history of myocardial infarction, angina pectoris, stroke or peripheral arterial occlusive disease), body mass index, and systolic and diastolic blood pressure (the mean of three measurements during one visit, performed with a mercury sphygmomanometer at the left arm, with the individual seated). The presence of hypertension was defined as a blood pressure > 140 mmHg, > 90 mmHg and/or use of antihypertensive medication. Hypercholesterolaemia was defined as a serum total cholesterol level > 6.5 mmol/l and/or the use of lipid-lowering

medication. Blood samples were taken in the fasting state. Plasma was separated and frozen immediately at –20°C for homocysteine and at –70°C for markers of endothelial function and inflammation, until analysis.

Measurement of markers of endothelial function and inflammatory activity

We determined vWf, soluble intercellular adhesion molecule-1 (sICAM-1), sVCAM-1, soluble E-selectin (ES), tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) as markers of endothelial function. Plasma vWf antigen was measured by an enzyme-linked immunosorbent assay (ELISA), with rabbit anti-vWf antigen as a capturing antibody and a peroxidase-conjugated rabbit anti-vWf antigen as a detecting antibody (Dako, Copenhagen, Denmark). The concentration of vWf was expressed as a percentage of the antigen concentration in normal pooled plasma, which is defined as 100%. The intra- and interassay variations were 2.3 and 3.8%, respectively. sICAM-1 and sVCAM-1 were assayed by ELISA methods (Diaclone, Besancon, France) with intra- and interassay coefficients of variation of 4.0 and 8.6% for sICAM-1 and 4.0 and 8.6% for sVCAM-1, respectively. Commercially available ELISA kits were used for the measurement of ES (R&D Systems, Minneapolis, MN), tPA antigen (Imulyse tPA, Biopool, Umeå, Sweden), and PAI-1 (Innotest PAI-1, Innogenetics, Zwijndrecht, Belgium).

Plasma concentrations of CRP and secretory phospholipase A_2 ($sPLA_2$) were measured as markers of inflammatory activity. CRP was measured with a highly sensitive in-house ELISA, with rabbit anti-CRP (Dako, Copenhagen, Denmark) as a capturing and tagging antibody, with intra- and interassay coefficients of variation of 3.8 and 4.7%, respectively. Antigen concentration of $sPLA_2$ were measured with an ELISA with two different monoclonal antibodies against human $sPLA_2$ (Oklahoma Medical Research Foundation, Oklahoma City, OK), which were used as coating and capturing antibodies, respectively. All markers were measured in duplicate.

Other measurements

Plasma total (free plus protein-bound) homocysteine (tHcy) was measured by HPLC with fluorescence detection. Intra- and interassay coefficients of variation were 2.1 and 5.1% respectively. Plasma total cholesterol and serum creatinine were measured with standard laboratory methods, as were urinary levels of creatinine and protein. Serum folate and vitamin B_{12} levels were determined by radioassay (ICN Pharmaceuticals, Costa Mesa, CA).

Statistical analysis

Variables were tested for normality and log-transformed if necessary. ANOVA was used to test differences between groups. When significant differences were found, pairwise comparisons were made using the Student's *t*-test. Pearson's test was used to assess correlation coefficients. Multiple linear regression analysis was performed to investigate the relationship between all markers of endothelial function and inflammatory activity as dependent factors, and creatinine clearance and other factors (tHcy, plasma total cholesterol and blood pressure) as independent variables, with forced entry of age, gender, smoking and prior CVD.

To explore further the association between creatinine clearance and endothelial function and inflammatory activity, we created mean SD scores for markers of endothelial function and inflammatory activity. This approach was used to reduce the influences of biological variability of separate measurements, and to reduce the number of associations to be investigated, as has been described by O'Brien [7]. The method has been used before by our group for evaluating markers of endothelial dysfunction, but has not been validated because of the lack of a gold standard for endothelial dysfunction [8]. For every individual, each variable was expressed as the SD of the difference from the population mean. The mean scores were calculated as the mean of these SDs. The endothelial function score was calculated as (vWf + sVCAM-1 + sICAM-1 + ES + PAI-1 + tPA)/6, and the inflammatory activity score as (CRP + $sPLA_2$)/2. Because sICAM-1 probably is a marker not only of endothelial function but also of inflammatory activity, we recalculated the inflammatory activity score also after addition of sICAM-1. Thus, high scores reflect high levels of endothelial dysfunction and inflammatory activity, respectively. A P value < 0.05 was considered to reflect statistical significance.

RESULTS

Table 1 shows baseline characteristics of the participants. There were no significant differences with regard to gender, body mass index, presence of hypercholesterolaemia, prior CVD, serum cholesterol, vitamin B_{12} and folic acid, and use of acetylsalicylic acid or a statin, among the four groups. Patients in the control group had a significantly lower mean age, blood pressure (both systolic and diastolic), plasma level of homocysteine and urinary excretion of protein compared to the patients in the other three groups. Otherwise, in the

control group, the prevalence of hypertension was significantly lower than that in the other three groups, as was the use of angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists. The prevalence of active smokers was higher in the control group. In one male subject, a very high plasma homocysteine level was found (94.6 μmol/l). This was shown to be caused by a vitamin B_{12} deficiency (serum level 65 pmol/l) due to pernicious anaemia. This subject was excluded from the analyses when plasma homocysteine was considered as a variable. In all other participants, vitamin B_{12} status was normal**.**

Table 1. Baseline characteristics

Variables	Q1 (Control) *n* = 20	Q2 *n* = 20	Q3 *n* = 20	Q4 *n* = 20
Creatinine clearance (ml/min/1.73 m^2)	100.3 ± 21.7 (83–163)	67.3 ± 8.6 (54–79)	40.0 ± 7.6 (28–52)	16.3 ± 5.4 (8–27)
Gender (male/female)	8/12	9/11	11/9	12/8
Age (years)	36.9 ± 8.6 (25–53)	54.3 ± 8.7 (40–66)*	49.5 ± 12.5 (23–68)*	55.1 ± 12.2 (31–73)*
Body mass index (kg/m2)	25.1 ± 3.4 (20.2–31.5)	26.3 ± 3.1 (21.7–32.6)	24.6 ± 3.9 (18.4–35.9)	24.0 ± 3.8 (18.4–33.2)†
Systolic blood pressure (mmHg)	120.6 ± 13.1 (102–149)	133.8 ± 17.3 (114–171)*	131.8 ± 19.6 (97–177)*	137.7 ± 10.9 (113–161)*
Diastolic blood pressure (mmHg)	69.3 ± 9.3 (58–93)	78.5 ± 8.0 (69-95)*	78.0 ± 9.5 (54–97)*	81.5 ± 6.9 (71–100)*
Hypertension (Yes/No)	5/15	15/5*	19/1*	20/0*†
ACEI or AII antagonist (Yes/No)	4/16	6/14	13/7*†	10/10*
Proteinuria (g/day)	0.096 (0.050–6.996)	0.145 (0.050–0.706)	0.825 (0.056–4.578)*†	1.337 (0.149–6.672)*†
Smoker (Yes/No)	14/6	7/13*	5/15*	6/14*
Serum folate (nmol/l)	13.3 (3.8–40.4)	16.8 (5.5–53.8)	12.4 (6.5–48.1)	13.5 (9.1–20.4)
Serum vitamin B_{12} (pmol/l)	309 (176–531)	286 (65–1009)	274 (140–395)	215 (140–611)
Plasma total homocysteine (μmol/l)	8.2 (5.3–17.0)	8.9 (5.3–94.6)	20.6 (6.8–66.4)*†	23.1 (13.7–32.2)*†
Plasma total cholesterol (mmol/l)	5.2 ± 1.4 (2.9–7.7)	5.4 ± 0.7 (4.0–6.7)	5.8 ± 1.6 (3.7–9.8)	5.5 ± 1.2 (2.0–6.7)
Hypercholesterolaemia (Yes/No)	3/17	4/16	10/10*†	7/13
Statin (Yes/No)	0/20	4/16*	4/16*	5/15*
Prior cardiovascular disease (Yes/No)	1/19	5/15	3/17	5/15
Acetylsalicylic acid (Yes/No)	1/19	4/16	4/16	2/18

Values are presented as mean ± SD (range) or median (range) for variables with a skewed distribution. Q, quartile; ACEI, angiotensin-converting enzyme inhibitor; AII antagonist, angiotensin II receptor antagonist. *P < 0.05 compared with Q 1; †P < 0.05 compared with Q 2.

Table 2 shows the plasma concentrations of the markers of endothelial function and inflammatory activity.

Table 2. Biochemical markers of endothelial function and inflammatory activity

Variables	Q1 (Control) *n* = 20	Q2 *n* = 20	Q3 *n* = 20	Q4 *n* = 20
vWf (%)	91 (42–232]	111 (57–250)*	159 (90–390)*†	173 (94–298)*†
sICAM-1 (ng/ml)	470 (225–1281]	562 (202–913)	539 (394–1088)	658 (358–1302)
sVCAM-1 (ng/ml)	1126 ± 338 (613–1983)	1080 ± 249 (673–1516)	1556 ± 444 (754–2376)*†	1796 ± 594 (1164–3469)*†
sES (ng/ml)	45 ± 21 (10–96)	48 ± 26 (20–129)	49 ± 28 (19–144)	80 ± 109 (11–516)
PAI-1 (ng/ml)	22 (6–224)	48 (7–196)*	29 (4–82)†	42 (3–124)
tPA (ng/ml)	9.2 ± 4.5 (4.0–22.2)	12.4 ± 3.8 (4.8–18.0)*	9.5 ± 5.4 (2.2–22.6)	10.4 ± 4.6 (3.8–17.9)
CRP (mg/l)	1.7 ± 2.5 (0.1–10.4)	2.3 ± 1.5 (0.3–10.4)	4.5 ± 11.1 (0.6–51.0)	5.0 ± 4.1 (0.3–14.4)*†
$sPLA_2$ (µg/l)	1.1 ± 3.9 (0.0–16.5)	1.9 ± 4.4 (0.0–16.4)	3.2 ± 9.5 (0.0–41.8)	6.7 ± 7.1 (0.0–23.7)*†

Values are presented as mean ± SD (range) or median (range) for variables with a skewed distribution. Q, quartile. *P < 0.05 compared with Q1; †P < 0.05 compared with Q2.

Table 3 shows the correlations of creatinine clearance with markers of endothelial function, inflammatory activity and conventional risk factors for CVD.

Table 3. Relation of creatinine clearance with markers of endothelial function and inflammatory activity, and conventional risk factors for cardiovascular disease

	Creatinine clearance (ml/min/1.73 m^2)	
Continuous variables	Correlation coefficient	P value
Endothelial function score	–0.43	< 0.001
Inflammatory activity score	–0.25	< 0.05
vWf*	–0.54	< 0.001
sICAM-1*	–0.21	0.06
sVCAM-1	–0.50	< 0.001
ES	–0.19	0.10
PAI-1*	–0.10	0.40
tPA	–0.06	0.61
CRP	–0.19	0.10
$sPLA_2$	–0.28	< 0.05
Age	–0.54	< 0.001
Body mass index	0.15	0.19
Systolic blood pressure	–0.36	< 0.01
Diastolic blood pressure	–0.44	< 0.001
Proteinuria*	–0.54	< 0.001
Serum folate*	–0.08	0.54
Serum vitamin B_{12}*	0.13	0.34
Plasma total homocysteine *	–0.61	< 0.001
Plasma total cholesterol	–0.19	0.08
Nominal variables	Yes	No	P value
Male gender	51.7 ± 34.3	60.3 ± 33.1	0.26
Hypertension	42.8 ± 24.9	93.1 ± 27.6	< 0.001
Smoker	69.0 ± 38.9	47.3 ± 27.1	< 0.01
Hypercholesterolaemia	46.3 ± 26.6	60.4 ± 36.0	0.08
Prior cardiovascular disease	44.5 ± 27.0	58.4 ± 34.8	0.16

Values are shown as Pearson correlation coefficients for continuous variables and as mean of the creatinine clearance ± SD for nominal variables. *Data logarithmically transformed.

Creatinine clearance was significantly related to the endothelial function score, vWf and sVCAM-1 (Figure 1). The relationships between creatinine clearance and the inflammatory activity score and $sPLA_2$ are shown in Figure 2. One subject showed very high inflammatory markers (CRP 51.0 mg/l and $sPLA_2$ 41.8 µg/l) without signs or symptoms of disease. When this patient was omitted from the analyses, inflammatory activity score ($r = –0.43$, $P < 0.001$), CRP ($r = –0.35$, $P < 0.01$) and $sPLA_2$ ($r = –0.34$, $P < 0.01$) correlated even more strongly with creatinine clearance. Plasma tHcy correlated negatively with creatinine clearance ($r = –0.61$, $P < 0.001$), plasma folate ($r = –0.34$, $P < 0.05$), and plasma vitamin B_{12} ($r = –0.24$, $P = 0.07$).

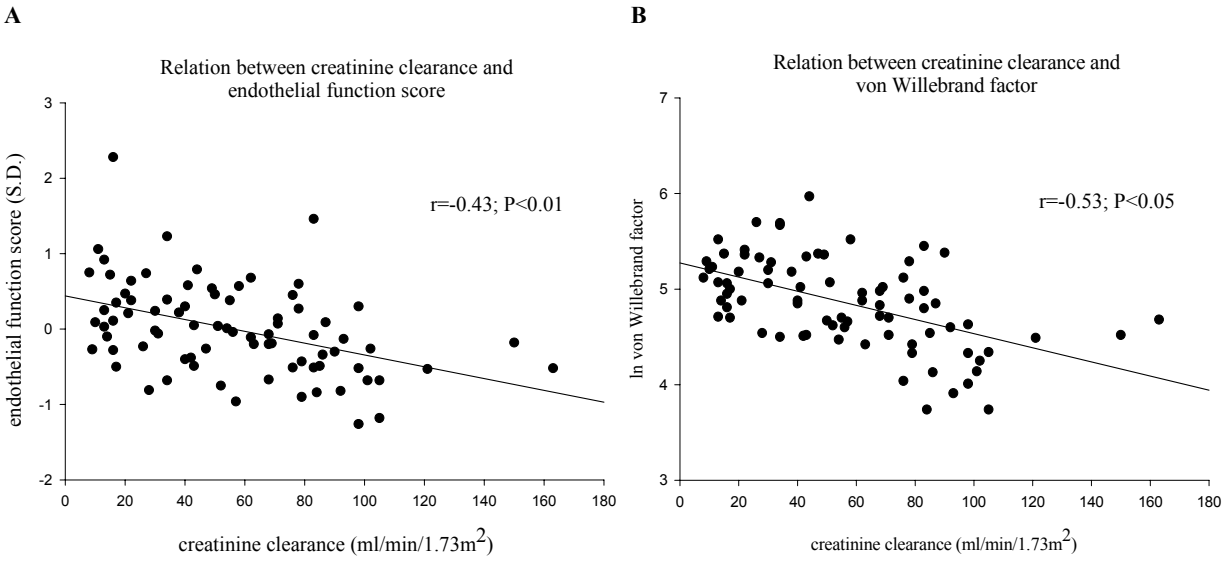

Figure 1. Relationship between (**A**) creatinine clearance and endothelial function score and (**B**) creatinine clearance and von Willebrand factor.

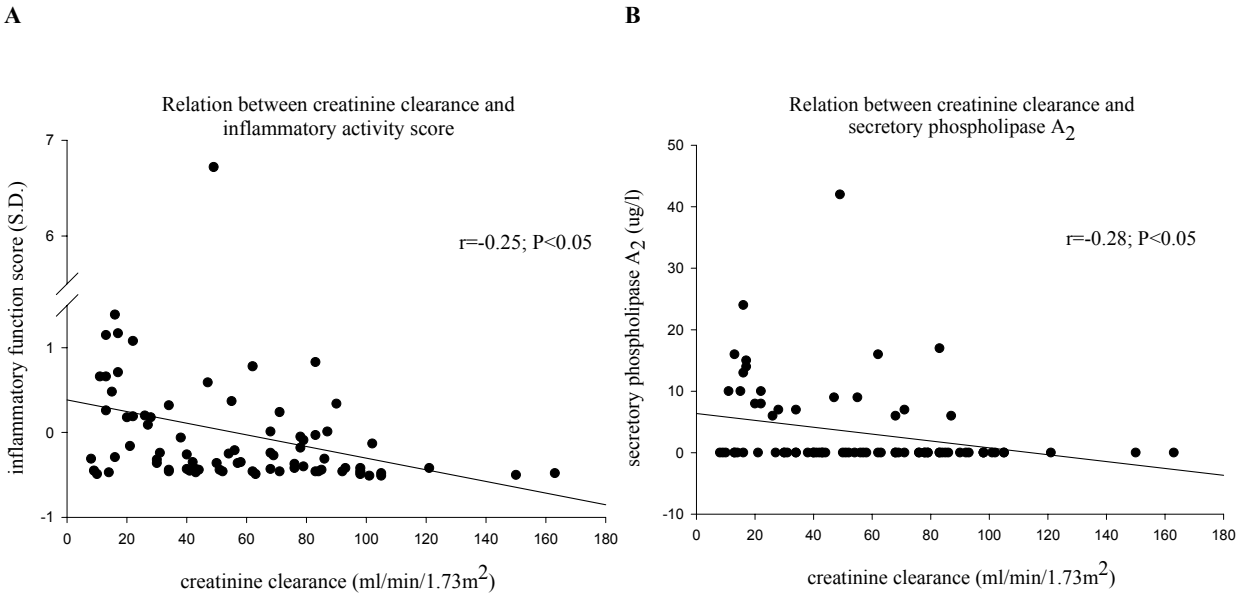

Figure 2. Relationship between (**A**) creatinine clearance and inflammatory activity score and (**B**) creatinine clearance and secretory phospholipase A_2.

Endothelial function

In the multivariate analysis (with forced entry of gender, age, smoking and prior CVD), creatinine clearance was a significant determinant of the endothelial function score [β (standardised) = –0.34, $P < 0.01$). After addition of systolic blood pressure to the model, the relationship between creatinine clearance and endothelial function score was somewhat attenuated (β = –0.27, $P < 0.05$). Substituting diastolic or mean blood pressure for systolic blood pressure did not materially change any of the results. After addition of plasma tHcy to the model, creatinine clearance was no longer a significant determinant of the endothelial function score (β = –0.05, $P = 0.74$). Plasma tHcy was independently associated with the endothelial function score (β = 0.28, $P < 0.05$). The presence of an underlying renal disease was not a significant determinant of the endothelial function score (β = 0.10, $P = 0.41$), nor were the separately entered diagnoses hypertensive nephrosclerosis (β = –0.17, $P = 0.23$), polycystic kidney disease (β = –0.03, $P = 0.81$) and primary glomerular disease (β = 0.19, $P = 0.12$).

When the individual markers of endothelial function were analysed separately, creatinine clearance was a significant determinant of vWf, sICAM-1 and sVCAM-1. Addition of systolic blood pressure to the model did not influence these relationships. When plasma tHcy was added to the model, creatinine clearance remained a significant determinant of vWf (β = –0.37, $P < 0.05$), but the strength of the associations between creatinine clearance and sVCAM-1 (β = –0.44, $P < 0.01$) and sICAM-1 (β = –0.33, $P < 0.05$) decreased (β = –0.22, $P = 0.17$ and β = –0.31, $P = 0.07$, respectively). Plasma tHcy was associated independently with sVCAM-1 (β = 0.37, $P < 0.01$). The association between creatinine clearance and vWf remained significant after addition of plasma total cholesterol to the model (β = –0.47, $P < 0.001$).

Inflammatory activity

Creatinine clearance was an independent determinant of the inflammatory activity score (β = –0.31, $P < 0.05$). However, after adjustment for systolic blood pressure, this association was no longer significant (β = –0.19, $P = 0.14$). Systolic blood pressure was independently associated with the inflammatory activity score (β = 0.41, $P < 0.001$). Addition of sICAM-1 to the inflammatory activity score did not materially change the results. The presence of an underlying renal disease was not a significant determinant of the inflammatory activity score (β = 0.04, $P = 0.76$), nor were the separately entered diagnoses hypertensive nephrosclerosis

(β = 0.20, P = 0.21), polycystic kidney disease (β = 0.00, P = 0.97) and primary glomerular disease (β = 0.02, P = 0.87).

Multivariate analysis of the individual markers of inflammatory activity showed that creatinine clearance was an independent determinant of $sPLA_2$ (β = –0.32, P < 0.05). The relationship with CRP was borderline significant (β = –0.25, P = 0.07). After adjustment for systolic blood pressure, both these associations were no longer significant (β = 0.18, P = 0.16 and β = –0.17, P = 0.22, respectively). Systolic blood pressure proved to be an independent determinant of plasma $sPLA_2$ and CRP (β = 0.46, P < 0.001 and β = 0.28, P < 0.05, respectively).

DISCUSSION

In this study, impaired renal function was associated with markers of endothelial dysfunction and increased inflammatory activity, as assessed by the scores derived from several different biochemical markers. The individual markers which showed the strongest associations with renal function were vWf, sVCAM-1, sICAM-1, $sPLA_2$ and CRP. We also showed, for the first time, that this effect is linear and is present already in patients with mild renal insufficiency.

The endothelial function score was no longer significantly associated with renal function when plasma tHcy was added as a factor in the multivariate model. The same was true for the relationship between sVCAM-1 and renal function, and plasma tHcy affected the association between endothelial function score and renal function probably by its relationship with sVCAM-1.

The relationship between renal function and sVCAM-1 has not been extensively investigated. In agreement with others, we found that plasma sVCAM-1 was elevated in patients with impaired renal function [6]. Urinary excretion of sVCAM-1 is very low in healthy persons and may rise in certain types of renal failure [9]. Decreased excretion of sVCAM-1 therefore does not explain the elevated plasma sVCAM-1 in individuals with reduced creatinine clearance.

The adhesion molecule sVCAM-1 is believed to play a crucial role in the development of atherosclerosis [10]. In the Hoorn Study it was shown prospectively that, among subjects with type 2 diabetes mellitus, the relative risk of cardiovascular mortality was 1.13 (95% confidence interval 1.07 to 1.20) per 100 ng/ml increase in sVCAM-1 [11].

The relationship between creatinine clearance and sVCAM-1 was abolished when plasma tHcy was added into the regression model. It is well known that plasma tHcy increases with declining renal function [12]. In our study, plasma tHcy was related independently not only to creatinine clearance, but also to plasma sVCAM-1 level. The most likely interpretation of this finding is that plasma tHcy is an intermediate in this relationship, i.e. that renal insufficiency leads to elevated plasma tHcy levels which, in turn, induce endothelial dysfunction reflected by elevated sVCAM-1 levels. This is supported by findings in a murine model, in which diet-induced hyperhomocysteinaemia resulted in enhanced atherosclerosis with increased expression of sVCAM-1 in the arterial wall [13]. In addition, a recent in vitro study showed that human aortic endothelial cells exhibited an increase of VCAM-1 expression when homocysteine was added to the medium [14]. An alternative interpretation is that homocysteine causes both renal insufficiency and endothelial dysfunction (i.e. confounds the association between creatinine clearance and sVCAM-1), but there is no firm evidence that homocysteine can in fact cause renal insufficiency.

The inverse relationship between creatinine clearance and plasma vWf level that we found was independent of conventional cardiovascular risk factors and plasma homocysteine. Thambyrajah et al. also observed elevated plasma vWf concentrations in 80 subjects with non-diabetic chronic renal failure [15]. In that study, however, the relationship between plasma vWf and renal function could not be defined accurately, probably as a result of the smaller range of creatinine clearance (14 to 54 ml/min). In another, smaller study, plasma vWf tended to increase with declining renal function [6]. We found that plasma vWf was already increased at an early stage of renal insufficiency (creatinine clearance < 91 ml/min). This increase in plasma vWf is probably not caused by diminished renal excretion because, in healthy persons, urinary vWf levels are undetectable [16]. Although it is possible that the metabolism of vWf is altered in renal insufficiency, we speculate that the rise in plasma vWf level is the result of endothelial dysfunction caused by renal insufficiency. The clinical importance of high plasma vWf levels is underlined by studies that have shown that plasma vWf is a predictor of microalbuminuria, CVD and mortality in both diabetic and non-diabetic patients [17].

Creatinine clearance was an independent predictor of the inflammatory activity score, but this relationship disappeared when adjusted for systolic blood pressure. The relationship between the inflammatory markers $sPLA_2$ and CRP, and blood pressure, however, is unclear. In the WOSCOP study, CRP significantly correlated with systolic blood pressure, while $sPLA_2$ did not [18]. In that study, the predictive value of CRP for coronary events was

attenuated when systolic blood pressure was included in the multivariate model, whereas the association between $sPLA_2$ and coronary events was not influenced [18]. The precise relationship between inflammatory activity and blood pressure deserves further investigation.

Renal function has been implicated as an independent cardiovascular risk factor, especially in individuals with essential hypertension, heart failure and coronary artery disease [19]. Our study may provide a clue why renal function may act as the "Cinderella of cardiovascular risk profile" [19]. Reduced glomerular filtration rate may lead to endothelial dysfunction and inflammatory activity, that can be detected by measurement of biochemical markers which have been shown to predict CVD. The cross-sectional character of our study does not allow any conclusion on the causality of these relationships. Furthermore, the pathophysiological mechanism by which reduction of glomerular filtration rate leads to dysfunction of the endothelium and to increased inflammatory activity cannot be derived from our data and remains to be elucidated. Our study population was heterogeneous concerning the underlying renal disorders. However, (the type of) renal disease was not a determinant of the markers of endothelial function and inflammatory activity. The high prevalence of hypertension in the groups with the lowest creatinine clearance is a common finding, for which we chose not to match, but to correct with a multivariate analysis. We found a possible role of homocysteine in the rise of sVCAM-1, and of blood pressure in the rise of $sPLA_2$. In addition, retention of uraemic toxins, e.g. advanced glycation end-products, or oxidative stress may contribute to these processes. This hypothesis could be tested in prospective trials with subjects with mild to moderate renal insufficiency using interventions which aim to improve endothelial function and lower the inflammatory state. It is of note that homocysteine-lowering treatment with folic acid did not result in amelioration of endothelium-dependent vasodilatation or plasma vWf in patients with predialysis renal failure [20]. In these studies, the effect of folic acid on sVCAM-1, however, was not investigated.

The clinical relevance of the present study may be that in individuals with even minor reductions of creatinine clearance, an aggressive approach should be adopted to prevent further decline of renal function, e.g. by intensive treatment of elevated blood pressure and proteinuria. Whether patients with mild renal failure should be treated with specific anti-inflammatory agents is currently unknown.

In summary, this cross-sectional study showed that impaired renal function per se was associated with endothelial dysfunction and increased inflammatory activity as assessed by plasma levels of vWf, sVCAM-1, sICAM-1, CRP and $sPLA_2$.

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Chapter 7

Advanced glycation end-product-peptides are associated with impaired renal function, but not with biochemical markers of endothelial dysfunction and inflammation, in non-diabetic individuals

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ABSTRACT

Background. Patients with end-stage renal disease as well as mild renal impairment have an increased risk for the development of cardiovascular disease. It has been suggested that advanced glycation end-products (AGEs) are involved in atherogenesis, possibly through induction of endothelial dysfunction and low-grade inflammation.

Methods. In a cross-sectional, single-centre study, we investigated four groups of twenty non-diabetic subjects with a creatinine clearance ranging from normal (> 90 ml/min per 1.73 m^2) to < 31 ml/min per 1.73 m^2. We measured AGE-peptides, markers of endothelial dysfunction (von Willebrand factor, soluble E-selectin, plasminogen activator inhibitor-1, tissue-type plasminogen activator, soluble vascular-cell adhesion molecule-1), and markers of inflammatory activity (soluble intercellular adhesion molecule-1, C-reactive protein, secretory phospholipase A_2). We constructed composite endothelial dysfunction and inflammatory activity Z-scores using these markers.

Results. AGE-peptides were independently related to creatinine clearance (standardized β –0.55; 95% confidence interval (CI) –0.77 to –0.34, P < 0.001). AGE-peptides were not independently related to the individual markers of endothelial dysfunction and inflammation, nor to the composite endothelial dysfunction Z-score (standardized β 0.08; 95% CI –0.14 to +0.30, P = 0.48) or the inflammatory activity Z-score (standardized β –0.05; 95% CI –0.25 to +0.16, P = 0.66).

Conclusions. Plasma concentrations of AGE-peptides are associated with creatinine clearance but not with biochemical markers of endothelial dysfunction and inflammatory activity in non-diabetic patients over a wide range of renal function. This suggests that the atherogenic effects of AGE-peptides in individuals with renal functional impairment are not mediated by endothelial dysfunction or inflammatory activity as estimated by the markers used.

INTRODUCTION

Patients with chronic kidney disease, end-stage renal disease as well as mild renal impairment, have an increased risk for the development of cardiovascular disease. Endothelial dysfunction and chronic low-grade inflammation may play a major role in atherogenesis and evidence accumulates that atherosclerosis may be an inflammatory disease in which immune mechanisms interact with metabolic risk factors [1]. A high blood concentration of advanced glycation end-products (AGEs), a heterogeneous group of compounds derived from the non-enzymatic reaction between glucose or other reducing sugars and proteins, has been suggested to be one of these metabolic factors [2].

AGEs may be involved in atherogenesis by interacting with specific receptors for AGE, which leads to the production of free oxygen radicals and subsequent release of cytokines [3]. In addition, AGEs have been found to accumulate in the vascular matrix where they are thought to disturb endothelial permeability and induce vessel-wall thickening [2]. Accumulation of AGEs in plasma has been described in diabetic and non-diabetic patients with end-stage renal disease [4]. Information about the relationship between creatinine clearance and AGEs in non-diabetic individuals with predialysis renal dysfunction is scarce and mainly derived from patients after kidney transplantation, where blood concentrations of AGEs normalized when normalization of renal function was achieved after kidney transplantation [5].

Resistance to complete enzymatic degradation of AGE-modified proteins leads to the formation of AGE-peptides. Measurement of AGE-peptides can be easily performed by a simple analytical procedure using spectrophotometry and spectrofluoroscopy as first described by Wróbel et al. [6]. Age-peptides have been shown to be related to renal function in diabetic patients with chronic kidney disease [7] and non-diabetic patients after kidney transplantation [5].

In the present study, we investigated the relationship between renal function, as estimated by the Cockcroft-Gault creatinine clearance, and AGE-peptides in non-diabetic individuals with a wide range of renal function. In addition, we aimed to investigate whether AGE-peptides are associated with biochemical markers of endothelial dysfunction and inflammatory activity.

SUBJECTS AND METHODS

Subjects

The study population has been previously described [8]. Briefly, 80 consecutive patients were recruited from the outpatient clinics of the Departments of Nephrology and Internal Medicine of the VU University Medical Centre. Exclusion criteria were age under 18 years or over 75 years, haemodialysis, peritoneal dialysis, renal transplantation, diabetes mellitus (ADA criteria), inflammatory diseases, WHO performance greater than 2, severe other disease (malignancy or liver disease) or use of folic acid, immunosuppressive drugs, penicillamine or anticonvulsant drugs. Patients were included until four groups of 20 subjects were formed with a creatinine clearance (calculated with the formula of Cockcroft and Gault) of 91 ml/min or higher, 61 to 90 ml/min, 31 to 60 ml/min and less than 31 ml/min per 1.73 m^2. This was done to assure a balanced representation throughout the whole spectrum of renal function. After correcting the creatinine clearance for body surface area, the group was divided into quartiles. In 65 (81%) of the 80 subjects a renal disease was diagnosed: hypertensive nephrosclerosis in 20, polycystic kidney disease in 15, primary glomerular disease in 14, urolithiasis in 4, non-active vasculitis in 4, chronic pyelonephritis in 2, Alport syndrome in 2 and other renal diseases in 4. Most of the 15 (19%) patients without renal disease were evaluated for chronic fatigue or treated for thyroidal illness. Of these 15 patients without renal disease 10 were included in the control group and 5 in the group with a creatinine clearance between 61 and 90 ml/min. Of each patient, data were collected with regard to age, cardiovascular medication (antihypertensive medication, platelet aggregation inhibitors and lipid-lowering drugs), smoking status (having smoked in the past year), presence of cardiovascular disease (history of myocardial infarction, angina pectoris, stroke or peripheral arterial occlusive disease), body mass index, and systolic and diastolic blood pressure (the mean of three measurements during one visit, performed with a mercury sphygmanometer at the left arm, with the individual seated). The presence of hypertension was defined as a systolic blood pressure > 140mmHg, a diastolic blood pressure > 90 mmHg and/or use of antihypertensive medication. Hypercholesterolaemia was defined as a serum total cholesterol level > 5.0 mmol/l and/or the use of lipid-lowering medication. Blood samples were taken in the fasting state. Plasma was immediately separated and frozen at –20 ^{o}C for homocysteine and at –70 ^{o}C for AGEs and markers of endothelial dysfunction and inflammation, until analysis.

The protocol was approved by the Ethics Committee of the VU University Medical Centre and all patients gave their informed consent.

Measurement of advanced glycation end-product-peptides

AGE-peptides were measured with a simple analytical procedure as described by Wróbel et al. [6] based on simultaneous detection of low-molecular-mass peptides and AGE-peptides with a flow system and two detectors connected on-line: spectrophotometrically for peptides (absorption at 280 nm) and spectrofluorometrically for AGE-peptides (λ_{ex} = 247 nm, λ_{em} = 440 nm). Briefly, plasma (40 µL) in microcentrifuge tubes was deproteinized with trichloroacetic acid (460 µL, 0.15 mol/l) and lipids were simultaneously extracted with chloroform (100 µL). The tubes were shaken vigorously and were centrifuged (10 min x 12000 g). Twenty µl of the aqueous layer was injected and fluorescence and absorption signals were measured. The ratio between fluorescence and absorption signal was calculated. AGE-peptide concentrations were determined with a proteinase K digest of AGE-bovine serum albumin (BSA) as calibrator (100% AGE-peptide calibrator). AGE-BSA was prepared by incubating BSA (50 g/l) with 0.5 mol/l glucose in 0.2 mol/l phosphate buffer (pH 7.4) for 42 days. After incubation, dialysis against phosphate buffered saline was carried out to remove unbound material. The AGE-peptide calibrator was obtained by hydrolysis of AGE-BSA (50 g/l) with proteinase K: 10 µl of proteinase K solution (8 g/l) was mixed with 90 µl of AGE-BSA and incubated for 24 h at 37 °C. In parallel, BSA was incubated with proteinase K to obtain a peptide calibrator (0% AGE-peptide calibrator). Mean AGE-peptide levels (± SD) in 44 healthy subjects were 10.6% ± 1.7% (of the difference in AGE-peptide concentration between the 100% and 0% AGE-peptide calibrator), which is comparable with the AGE-peptides amounts measured in healthy subjects by Wróbel et al. [6]. Within-day and between-day coefficients of variation were < 3% at an AGE-peptide concentration corresponding to that in healthy subjects.

Other measurements

We determined plasma concentrations of von Willebrand factor (vWf), soluble vascular-cell adhesion molecule-1 (sVCAM-1), soluble E-selectin (sE-selectin), tissue-type plasminogen activator (tPA), and plasminogen activator inhibitor-1 (PAI-1) as markers of endothelial dysfunction, and soluble intercellular adhesion molecule-1 (sICAM-1), C-reactive protein (CRP) and secretory phospholipase A_2 ($sPLA_2$) as markers of inflammatory activity. All markers were measured in duplicate. In addition, plasma total (free plus protein bound)

homocysteine (tHcy) and total cholesterol, serum creatinine, and urinary levels of creatinine and protein were determined. All measurement techniques have been described previously [8].

Statistical analysis

All analyses were performed with SPSS, version 11.5 (SPSS Inc, Chicago, IL, U.S.A.). Variables are presented as mean ± standard deviation, number with percentage of total, or, in case of a skewed distribution, median with interquartile range. Analysis of variance (ANOVA) was used to test differences between groups. Pearson's test was used to asses bivariate correlations. To study whether AGE-peptides were related to creatinine clearance, body mass index, blood pressure, proteinuria and blood levels of total cholesterol, total homocysteine and markers of endothelial dysfunction and inflammatory activity, all regression analyses were performed with adjustment for age. In addition, to explore whether AGE-peptides were independently related to creatinine clearance, markers of endothelial dysfunction and markers of inflammatory activity, regression analyses were performed with five additional models. In the first model, multivariate analysis was performed with adjustment for age, sex, body mass index, diastolic blood pressure, the presence of cardiovascular disease and smoking status. In model 2 to 5, sequential adjustments were performed for plasma total cholesterol, plasma total homocysteine, and estimates of endothelial dysfunction and inflammatory activity, respectively. When necessary, variables were log-transformed for a better fit in linear regression analyses.

Because markers of endothelial dysfunction and inflammatory activity show marked intra-individual (day-to-day) variation and because we measured these markers only once, the associations (if any) of endothelial dysfunction and inflammatory activity with other variables will tend to be underestimated. Therefore, we created mean standard deviation (Z-)scores for markers of endothelial dysfunction and inflammatory activity, and used these in regression analyses as described below. For every individual, each variable was expressed as standard deviations of difference from the population mean. The mean Z-scores for endothelial dysfunction and inflammatory activity were calculated as the mean of these standard deviation scores as follows: endothelial dysfunction Z-score = (vWf + sVCAM-1 + sE-selectin + PAI-1 + tPA)/5, and inflammatory activity Z-score = (CRP + sICAM-1 + $sPLA_2$)/3. Thus, high scores reflect high levels of endothelial dysfunction and inflammatory activity, respectively.

A P-value < 0.05 was considered to reflect statistical significance.

RESULTS

Table 1 shows baseline characteristics of the participants according to quartiles of creatinine clearance.

Table 1. Baseline characteristics*

Variables	Q1 N=20	Q2 N=20	Q3 N=20	Q4 N=20	P value (trend)
Creatinine clearance (ml/min per 1.73 m^2)†	100 ± 22	67 ± 9	40 ± 8	16 ± 5	< 0.001
AGE-peptides (%)	10.0 (8.1–11.5)	10.5 (9.5–15.9)	14.7 (10.1–27.7)	32.9 (16.6–37.8)	< 0.001
Male sex (N [%])	8 [40]	9 [45]	11 [55]	12 [60]	0.59
Age (years)	37 ± 9	54 ± 9	50 ± 13	55 ± 12	< 0.001
Current smoker (N [%])	14 [70]	7 [35]	5 [25]	6 [30]	< 0.05
Prior cardiovascular disease (N [%])	1 [5]	5 [25]	3 [15]	5 [25]	0.29
Body mass index (kg/m2)	25.1 ± 3.4	26.3 ± 3.1	24.6 ± 3.9	24.0 ± 3.8	0.22
Systolic blood pressure (mmHg)	121 ± 13	134 ± 17	132 ± 20	138 ± 11	< 0.01
Diastolic blood pressure (mmHg)	69 ± 9	78 ± 8	78 ± 9	81 ± 7	< 0.001
Pulse pressure (mmHg)	51 (46–56)	52 (47–63)	49 (44–65)	57 (52–60)	0.44
Hypertension (N [%])‡	5 [25]	15 [75]	19 [95]	20 [100]	< 0.001
Plasma total cholesterol (mmol/l)	5.2 ± 1.4	5.4 ± 0.7	5.8 ± 1.6	5.5 ± 1.2	0.46
Hypercholesterolaemia (N [%])§	8 [40]	16 [80]	13 [65]	16 [20]	< 0.05
Plasma total homocysteine (µmol/l)	8.2 (7.2–11.4)	8.9 (6.7–10.9)	20.6 (11.5–25.0)	23.1 (19.1–27.1)	< 0.001
Proteinuria (g/day)	0.096 (0.050–0.378)	0.145 (0.104–0.341)	0.825 (0.287–1.855)	1.337 (0.548–2.096)	< 0.001
Von Willebrand factor (%)	91 (62–118)	111 (88–145)	159 (103–211)	173 (134–211)	< 0.001
Soluble E-selectin (ng/ml)	45 ± 21	48 ± 26	49 ± 28	80 ± 109	0.20
Plasminogen activator inhibitor-1 (ng/ml)	22 (12–34)	48 (28–83)	29 (12–50)	42 (18–62)	0.10
Tissue-type plasminogen activator (ng/ml)	9.2 ± 4.5	12.4 ± 3.8	9.5 ± 5.4	10.4 ± 4.6	0.13
Soluble VCAM-1 (ng/ml)	1126 ± 338	1080 ± 249	1556 ± 444	1796 ± 594	< 0.001
Endothelial dysfunction score (Z-score)¶	-0.41 ± 0.56	-0.02 ± 0.46	0.05 ± 0.56	0.38 ± 0.68	< 0.01
Soluble ICAM-1 (ng/ml)	470 (419–689)	562 (417–669)	539 (477–688)	658 (547–757)	0.17
C-reactive protein (mg/l)	1.7 ± 2.5	2.3 ± 1.5	4.5 ± 11.1	5.0 ± 4.1	0.26
Secretory phospholipase A_2 (µg/l)	1.1 ± 3.9	1.9 ± 4.4	3.2 ± 9.5	6.7 ± 7.1	< 0.05
Inflammatory activity score (Z-score)‖	-0.26 ± 0.50	-0.20 ± 0.47	0.08 ± 1.23	0.38 ± 0.55	< 0.05

Values for continuous variables are presented as mean ± SD or median (interquartile range) for variables with a skewed distribution. Analysis of variance (ANOVA) was used for calculation of P values for trend. Abbreviations: Q, quartile of creatinine clearance; AGE, advanced glycation end-product; VCAM-1, vascular adhesion molecule-1; ICAM-1; intercellular adhesion molecule-1.

*Adapted from a previous description of this study population [8]; †Estimated with the Cockcroft-Gault formula; ‡Systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg and/or the use of antihypertensive medication; §Plasma total cholesterol concentration > 5.0 mmol/l and/or the use of cholesterol lowering medication; ¶Calculated as the mean of the standard deviation scores as follows: (von Willebrand factor + soluble E-selectin + plasminogen activator inhibitor-1 + tissue-type plasminogen activator + soluble vascular adhesion molecule-1)/5; ‖Calculated as the mean of the standard deviation scores as follows: (Soluble intercellular adhesion molecule-1 + C-reactive protein + secretory phospholipase A_2)/3.

Figure 1 shows the significant inverse relationship between AGE-peptides and creatinine clearance. The age-adjusted relations between AGE-peptides and (potential) risk factors for cardiovascular disease are presented in Table 2. Age itself did not significantly correlate with AGE-peptides (r = 0.19, P = 0.10).

Multivariate analyses (Table 3) showed that, after adjustment for age, sex, body mass index, diastolic blood pressure, prior cardiovascular disease and smoking, creatinine clearance was independently associated with AGE-peptides. Sequential addition of total cholesterol, plasma total homocysteine, endothelial dysfunction score and plasma inflammatory activity score did not materially change this relationship. Besides creatinine clearance, none of the independent variables was significantly associated with AGE-peptides. When, in models 4 and 5, adjustment for the separate (instead of the Z-scores) markers for endothelial dysfunction and inflammation was performed, the relationship between creatinine clearance and AGE-peptides was also not materially affected (data not shown). AGE-peptides were not independently related to vWf (standardized β –0.04; 95% CI –0.25 to +0.18, P = 0.74), sE-selectin (standardized β 0.02; 95% CI –0.17 to +0.21, P = 0.81), PAI-1 (standardized β 0.15;

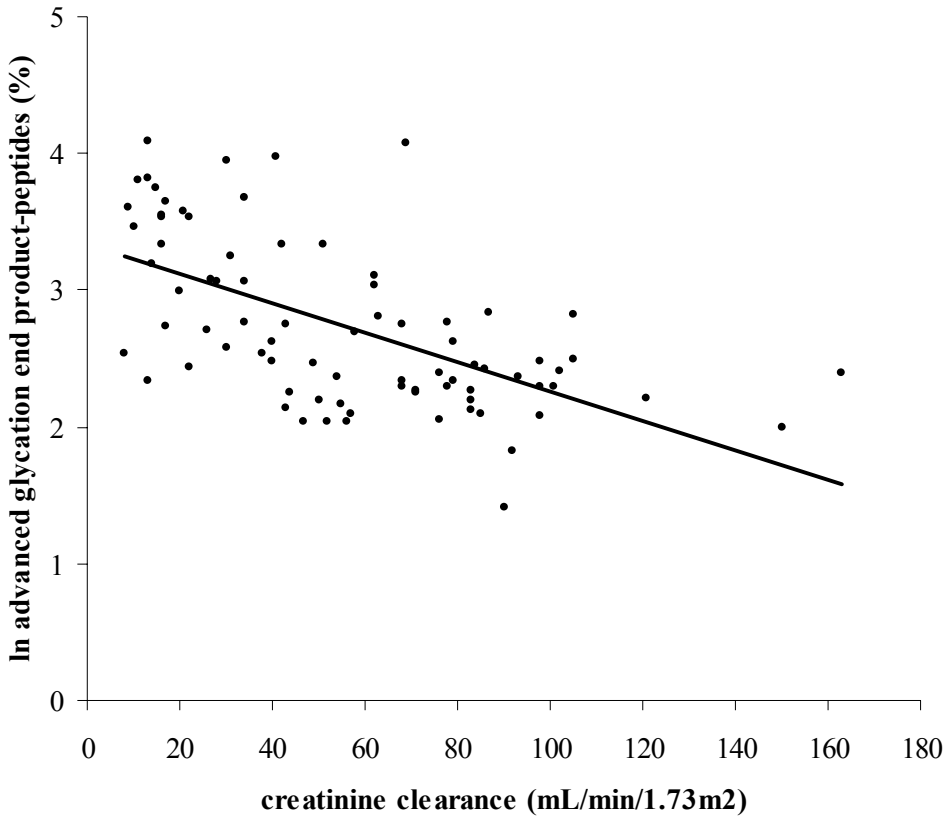

Figure 1. Relation between the natural logarithm of the plasma concentration of advanced glycation end-product-peptides and creatinine clearance (n = 80). Pearson's correlation coefficient: –0.59 (P < 0.001).

95% CI –0.06 to +0.35, P = 0.16), tPA (standardized β 0.08; 95% CI –0.12 to +0.27, P = 0.45), sVCAM-1 (standardized β –0.01; 95% CI –0.17 to +0.16, P = 0.95), or the endothelial dysfunction Z-score (standardized β 0.08, 95% CI –0.14 to +0.30, P = 0.48). Nor were AGE-peptides significantly related to sICAM-1 (standardized β 0.05, 95% CI –0.15 to 0.26, P = 0.62), CRP (standardized β –0.12; 95% CI –0.29 to +0.06, P = 0.23) and $sPLA_2$ (standardized β –0.02, 95% CI –0.22 to +0.18, P = 0.84), or to the inflammatory activity Z-score (standardized β –0.05; 95% CI –0.25 to 0.16, P = 0.66).

Tabel 2. Relation between advanced glycation end-product-peptides and risk factors for cardiovascular disease

	Advanced glycation end-product-peptides (%)*		P value
Continuous variables	Standardized β	(95% confidence interval)	
Creatinine clearance (ml/min per 1.73m^2)†	-0.70	(-0.92 – -0.48)	< 0.001
Body mass index (kg/m2)	-0.08	(-0.30 – 0.14)	0.47
Systolic blood pressure (mmHg)	0.32	(0.11 – 0.53)	< 0.01
Diastolic blood pressure (mmHg)	0.43	(0.21 – 0.64)	< 0.001
Pulse pressure (mmHg)*	0.12	(-0.11 – 0.35)	0.32
Plasma total cholesterol (mmol/l)	0.15	(-0.07 – 0.37)	0.20
Plasma total homocysteine (µmol/l)*	0.42	(0.22 – 0.62)	< 0.001
Proteinuria (g/day)*	0.36	(0.12 – 0.59)	< 0.01
Von Willebrand factor (%)*	0.31	(0.09 – 0.53)	< 0.01
Soluble E-selectin (ng/ml)	0.13	(-0.06 – 0.32)	0.24
Plasminogen activator inhibitor-1 (ng/ml)*	0.17	(-0.05 – 0.39)	0.15
Tissue-type plasminogen activator (ng/ml)	0.07	(-0.16 – 0.29)	0.57
Soluble vascular adhesion molecule-1 (ng/ml)	0.27	(0.11 – 0.44)	< 0.05
Endothelial dysfunction score (Z-score)‡	0.34	(0.11 – 0.57)	< 0.01
Soluble intercellular adhesion molecule-1 (ng/ml)*	0.24	(0.03 – 0.45)	< 0.05
C-reactive protein (mg/l)	0.01	(-0.21 – 0.23)	0.93
Secretory phospholipase A_2 (µg/l)	0.17	(-0.05 – 0.39)	0.12
Inflammatory activity score (Z-score)§	0.18	(-0.13 – 0.30)	0.11

Linear regression analysis of age-adjusted relations between advanced glycation end-product (AGE)-peptides and continuous variables.

*Data are logarithmically transformed; †Estimated with the Cockcroft-Gault formula; ‡Calculated as the mean of the standard deviation scores as follows: (von Willebrand factor + soluble E-selectin + plasminogen activator inhibitor-1 + tissue-type plasminogen activator + soluble vascular adhesion molecule-1)/5; §Calculated as the mean of the standard deviation scores as follows: (Soluble intercellular adhesion molecule-1 + C-reactive protein + secretory phospholipase A_2)/3.

Table 3. Multiple regression analysis of the relation between advanced glycation end-product-peptides and creatinine clearance

		Advanced glycation end-product-peptides*		
Model	Creatinine clearance†	Standardized β	(95% confidence interval)	P value
1	Age, sex, body mass index, diastolic blood pressure, prior CVD, current smoking	-0.55	(-0.77 – -0.34)	< 0.001
2	Model 1 + plasma total cholesterol	-0.56	(-0.77 – -0.34)	< 0.001
3	Model 1 + plasma total homocysteine*	-0.52	(-0.84 – -0.19)	< 0.001
4	Model 1 + endothelial dysfunction score (Z-score)‡	-0.54	(-0.75 – -0.32)	< 0.001
5	Model 1 + inflammatory activity score (Z-score)§	-0.56	(-0.78 – -0.35)	< 0.001

Abbreviation: CVD, cardiovascular disease.

*Data are logarithmically transformed; †Estimated with the Cockcroft-Gault formula; ‡Calculated as the mean of the standard deviation scores as follows: (von Willebrand factor + soluble E-selectin + plasminogen activator inhibitor-1 + tissue-type plasminogen activator + soluble vascular adhesion molecule-1)/5; §Calculated as the mean of the standard deviation scores as follows: (Soluble intercellular adhesion molecule-1 + C-reactive protein + secretory phospholipase A_2)/3.

Additional analysis

As age, sex, body weight and height are part of the formula for calculation of the body surface adjusted creatinine clearance, all models were tested without these variables. This did not materially influence our results. For example, in models 4 and 5, the standardized βs of the relations between creatinine clearance and AGE-peptides, without adjustment for sex, age, and body mass index, were –0.45 (95% CI –0.66 to –0.23, $P < 0.001$) and –0.46 (95% CI –0.68 to –0.25, $P < 0.001$), respectively (other data not shown).

DISCUSSION

This study shows that plasma concentrations of AGE-peptides were independently related to creatinine clearance in non-diabetic individuals over a wide range of renal function. AGE-peptides were not independently related to endothelial dysfunction and inflammatory activity, as estimated by a panel of markers.

The relation between renal function on the one hand and AGEs as well as AGE-peptides on the other hand has been mainly investigated and demonstrated in patients with diabetes mellitus [9], end-stage renal disease [10] and after kidney transplantation [5]. Sharp et al. found a positive relationship between serum creatinine and AGE-peptides in 106 healthy individuals with an apparently normal renal function (median serum creatinine 90 μmol/l, range 42 to 150 μmol/l) [7]. The present study, using a more accurate assessment of renal function, confirms this relationship in the full range from normal to predialysis renal function. The most likely mechanism for the accumulation of AGEs and AGE-peptides seems to be a decreased renal excretion [11], although contribution of a disturbed elimination of AGEs in the liver [12] or increased de novo generation of AGEs [13] cannot be excluded.

The relation between AGEs (or AGE-modified peptides) and cardiovascular complications is still under examination. The widespread belief that AGEs contribute to the development of cardiovascular disease [2] was initially based on animal studies that demonstrated properties of AGEs to induce vasoconstriction, complement activation, cytokine release, procoagulant effects and oxidative stress [3]. Furthermore, in a study in hemodialysis patients, alterations in left ventricular geometry were independently related to plasma concentration of the AGE pentosidine [14]. Clinical trials with inhibitors of AGE-formation (aminoguanidine) and cross-link breakers (ALT-711) have confirmed the potential role of AGEs in the development of vascular complications in diabetic patients [15]. So far, however, prospective studies in

patients with chronic kidney disease and/or diabetes have not been able to link high AGEs to cardiovascular morbidity and mortality [10].

We did not find an independent relationship between blood levels of high serum AGE-peptides and biomarkers of endothelial dysfunction and inflammatory activity. With respect to endothelial dysfunction, in vitro studies have shown that exposure of endothelial cells to AGEs enhances vascular expression of VCAM-1 and sE-selectin [16]. In diabetic patients, high serum AGEs (the major AGEs N^{ε}-(carboxymethyl)lysine [CML] and N^{ε}-(carboxyethyl)lysine [CEL]) were associated with high plasma levels of sVCAM-1, vWf and soluble thrombomodulin [9]. An association between high serum AGEs and impaired endothelium-dependent vasodilatation could be demonstrated in diabetic patients [17], which was not the case in end-stage renal disease [18].

With respect to inflammatory activity, expression of the receptor for AGE on peripheral blood monocytes has been shown to be associated with plasma levels of CRP and tumour necrosis factor alpha in patients with chronic kidney disease [19]. However, as in our study, a relationship between blood levels of AGE-peptides and CRP could also not be demonstrated in patients with end-stage renal disease [10] and diabetes [9].

This study has some limitations. The use of the AGE-peptides as reflection of AGEs in plasma requires some discussion. Several AGEs, such as argpyrimidine and pentosidine, exhibit characteristic fluorescence. Based on this, a novel simple analytical procedure to measure AGE-peptides with on-line spectrophotometric and spectrofluorometric detection was developed [6]. This technique has been validated with an AGE-Elisa [6], and we demonstrated a strong correlation of AGE-peptides with the well-characterized non-fluorescent AGEs CML and CEL, indicating the specificity of this technique for the quantification of AGEs [20]. However, the fluorophores as determined in this assay are unknown and the exact specificity of the assay needs to be further determined.

In addition, due to the limited number of patients and the variety of renal diseases, the possibility of a relationship between AGE-peptides and markers of endothelial function and inflammatory activity cannot be unequivocally excluded, especially in subgroups. The intra-individual variability of the markers of endothelial function and inflammatory activity was not assessed in our study, but the use of a composite Z-score of these markers should have lessened a masking effect (if any) on the associations that were studied. Of course, the cross-sectional nature of this study does not allow conclusions on the relationship between renal and cardiovascular outcomes.

In conclusion, plasma concentrations of AGE-peptides are associated with creatinine clearance but not with biochemical markers of endothelial dysfunction and inflammatory activity in non-diabetic patients with a wide range of predialysis renal function. Therefore, the supposed atherogenic effect of AGEs seems not to be mediated by endothelial dysfunction or chronic low-grade inflammation, as reflected by vWf, sVCAM-1, sE-selectin, PAI-1 and tPA, and CRP, sICAM-1 and $sPLA_2$, respectively. Thus, our study does not support the use of these biomarkers of endothelial dysfunction and inflammatory activity as intermediary end points of AGE-peptide-reducing interventions aiming for cardiovascular risk reduction.

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Chapter 8

Endothelial dysfunction contributes to renal function-associated cardiovascular mortality in a population with mild renal insufficiency: The Hoorn Study

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ABSTRACT

Mildly impaired renal function is associated with cardiovascular morbidity and mortality. There are indications that endothelial dysfunction and/or chronic inflammation, which play an important role in atherothrombosis, are present in early stages of renal insufficiency. This study investigated whether and to which extent endothelial dysfunction and inflammation were related to renal function and contributed to renal-function-associated cardiovascular mortality in a population-based cohort (n = 613), aged 50 to 75 yr, that was followed with a median duration of 12.5 yr. During follow-up, 192 individuals died (67 of cardiovascular causes). At baseline, renal function was estimated with serum creatinine, the Cockcroft-Gault formula, and the Modification of Diet in Renal Disease equation of glomerular filtration rate (eGFR). Endothelial function was estimated by plasma von Willebrand factor, soluble vascular cell adhesion molecule-1, and the urinary albumin-creatinine ratio. Inflammatory activity was estimated by plasma C-reactive protein and soluble intercellular adhesion molecule-1. Renal function was mildly impaired (mean eGFR 68 ± 12 ml/min per 1.73 m^2) and independently associated with von Willebrand factor [standardized β –0.09; 95% confidence interval (CI) –0.18 to –0.002; $P < 0.05$], soluble vascular cell adhesion molecule-1 (standardized β –0.14; 95% CI –0.22 to –0.05; $P < 0.01$) and albumin-creatinine ratio (standardized β –0.15; 95% CI –0.23 to –0.08; $P < 0.001$) but not with markers of inflammatory activity. Renal function was inversely associated with cardiovascular and all-cause mortality. The relative risk of cardiovascular mortality but not all-cause mortality associated with renal function decreased from 1.22 to 1.12 per 5 ml/min per 1.73 m^2 decrease of eGFR after adjustment for markers of endothelial dysfunction. In conclusion, endothelial dysfunction was related to renal function and contributed to the excess in cardiovascular mortality in this population-based cohort with mild renal insufficiency.

INTRODUCTION

Chronic kidney disease is a prevalent health problem, involving more than 10% of the general population of the United States [1]. Chronic kidney disease, especially end-stage renal disease, is strongly associated with the occurrence of cardiovascular disease [2]. As recently demonstrated, even mildly impaired renal function is associated with cardiovascular morbidity [3,4] and mortality [3,5]. Although chronic kidney disease is associated with several risk factors for cardiovascular disease, such as male gender, older age, hyperhomocysteinemia, hypertension, smoking, diabetes, obesity, and pre-existing cardiovascular disease, the excess cardiovascular risk in chronic kidney disease is not fully explained by this clustering of conventional risk factors.

In atherogenesis, endothelial dysfunction and inflammation are important and interrelated early steps [6]. Indeed, several biochemical markers of endothelial dysfunction and inflammatory activity have been shown to be independent risk factors of cardiovascular morbidity and mortality [7–11]. It is unknown whether the excess cardiovascular risk in chronic kidney disease is (partially) attributable to endothelial dysfunction and inflammation. There is evidence that a decreased glomerular filtration rate (GFR) is associated with endothelial dysfunction as well as inflammatory activity [12–14]. However, there is little evidence that a low GFR is associated with endothelial dysfunction and inflammation in the general population and, if so, whether any such associations can explain the association of a low GFR with risk for cardiovascular disease.

In view of these considerations, we investigated, in a prospective, population-based study (1) whether estimates of GFR were cross-sectionally associated with markers of endothelial dysfunction and inflammatory activity at the baseline examination and (2) whether endothelial dysfunction and inflammatory activity were involved in the excess mortality associated with a low GFR.

MATERIALS AND METHODS

Participants

The study population consisted of an age-, gender-, and glucose tolerance-stratified sample of the Hoorn Study, a population-based study of glucose tolerance and other cardiovascular

risk factors in a 50- to 75-yr-old general white population conducted from 1989 to 1992, as described previously [15].

Briefly, 2484 people (71% of those invited) participated. All participants, except for those who had previously diagnosed diabetes and were treated with oral glucose-lowering agents or insulin, underwent an oral glucose tolerance test (OGTT) according to the World Health Organization guidelines [16]. For reasons of efficiency, participants with a 2-h postload glucose ≥ 7.5 mmol/L, all participants with type 2 diabetes, and a random sample of participants with a 2-h postload glucose < 7.5 mmol/l stratified by age and gender were invited within four wk for a second visit to investigate glucose intolerance-related complications (709 invited, 631 [89%] of whom participated).

These participants underwent a second OGTT (except those who already used blood glucose-lowering agents; n = 67). On the basis of the mean of the two OGTT, glucose tolerance status was divided into three categories according to the 1999 World Health Organization criteria: Normal glucose metabolism, impaired glucose metabolism, and type 2 diabetes [17]. Participants in our study population thus represented a stratified random sample of all participants in the initial cohort.

The Hoorn Study was approved by the Ethical Committee of the VU University Medical Center. Informed consent was obtained from all participants.

Baseline laboratory and clinical assessments

C-reactive protein (CRP) and soluble intercellular adhesion molecule-1 (sICAM-1) were measured as markers of inflammatory activity, and soluble vascular cell adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWf), and microalbuminuria were measured as markers of endothelial dysfunction. After an overnight fast, blood was drawn from an antecubital vein. We measured blood concentrations of vWf, sVCAM-1, CRP, sICAM-1, total (free plus bound) homocysteine, glucose, creatinine, albumin, urea nitrogen, and lipids, as described elsewhere [10,15]. The urinary albumin-creatinine ratio (ACR) was calculated.

Creatinine clearance was estimated (eCC) by the Cockcroft-Gault formula [(140-age) × (1.23 × body weight/creatinine), amplified by 0.85 if female] [18]. Glomerular filtration rate was estimated (eGFR) by the Modification of Diet in Renal Disease (MDRD) equation [170 × (creatinine)$^{-0.999}$ × (age)$^{-0.176}$ × (serum urea nitrogen)$^{-0.170}$ × (albumin)$^{0.318}$ × (0.762 if patient is female) (1.180 if patient is black)] (MDRD equation is given in traditional units. To convert to International System units, multiply creatinine in mg/dl by 88.4, urea in mg/dl by 0.357, and albumin in g/dl by 10.) [19]. Estimated CC and eGFR were expressed in ml/min per 1.73 m^2

body surface area. Blood pressure was measured as the mean of four measurements performed on two different occasions, using a random-zero sphygmomanometer under standardized conditions. Participants were classified as current cigarette smokers or nonsmokers. Body mass index (BMI) and waist-to-hip ratio were calculated as described elsewhere [15]. Cardiovascular disease was defined as coronary artery disease, cerebrovascular disease, and/or peripheral arterial disease.

We excluded individuals for whom no data were available for calculation of the renal function estimates (n = 18), leaving 613 individuals available for analysis. From these individuals, data on vWf and sVCAM-1 were missing in 21, data on ACR in 23, and data on CRP and sICAM-1 in 23.

Follow-up

Data on the participants' vital status on January 1, 2004, were collected from the mortality register of the municipality of Hoorn. Of 51 participants who had moved out of town, information was obtained from the local municipalities. Of the 613 included participants, one was lost to follow-up. For the other 612 participants, we determined whether death had occurred during follow-up and, if so, the date when death occurred. For all participants who had died, the cause of death was extracted from the medical records of the general practitioner and the hospital of Hoorn and classified according to the of the International Classification of Diseases, Ninth Revision [20]. Cardiovascular mortality was defined as codes 390 to 459.

Statistical analyses

All analyses were performed with the SPSS, version 11.5 (SPSS Inc., Chicago, IL). Variables are presented as mean ± standard deviation (SD), number (percentage of the total), or, in case of a skewed distribution, the median and the interquartile range.

For descriptive purposes, the group was divided in tertiles according to the eGFR. P value for trend over tertiles was calculated with univariate analysis of variance (ANOVA) for continuous variables and with χ^2 test for binary variables. Pearson test was used to assess correlation coefficients.

To study whether biochemical markers of endothelial dysfunction and inflammatory activity were related independently to estimates of renal function, regression analyses were performed with three different models. In the first model–because of the stratification procedure–multivariate regression analysis was performed with adjustment for age, gender, and glucose tolerance status. In the second model, we additionally adjusted for potential

confounders, i.e., prior cardiovascular disease, systolic blood pressure, current smoking, waist-to-hip ratio, total cholesterol, and homocysteine. Finally, we adjusted the associations between renal function and endothelial dysfunction markers for markers of inflammation, and vice versa. Variables that did not have a normal distribution of the residuals (vWf, ACR and CRP) were transformed into their natural logarithm (ln) for a better fit of the data. Results are described as standardized β with 95% confidence intervals (CI).

Multivariate Cox regression analyses were performed to assess whether the associations of renal function with all-cause mortality and cardiovascular mortality were independent of potential confounding or mediating factors.

Adjustment for endothelial function was performed by adding each marker of endothelial dysfunction separately to the model and by adding vWf, sVCAM-1, and ACR simultaneously. Because microalbuminuria has been shown previously to have a heterogeneous association with endothelial dysfunction in the Hoorn Study [21,22], we also adjusted for sVCAM-1 and vWf only. In an additional analysis, sICAM-1 was added to vWf, sVCAM-1, and ACR, because it has been suggested that sICAM-1 may reflect endothelial dysfunction as well as inflammatory activity. Adjustment for inflammatory activity was performed by adding CRP and sICAM-1 (separately and simultaneously) to the model.

Results are described as relative risks (RR; hazard ratios) with 95% CI associated with a decrease in eGFR and eCC of 5 ml/min per 1.73 m^2 and increase in serum creatinine concentration of 5 μmol/L. Two-sided $P < 0.05$ was considered to reflect statistical significance.

RESULTS

Table 1 shows the baseline characteristics of the study population according to tertiles of eGFR. In the whole study population, eGFR was 68 ± 12 ml/min per 1.73 m^2, eCC was 74 ± 17 ml/min per 1.73 m^2, and serum creatinine was 92 ± 19 μmol/L.

Table 1. Baseline characteristics of all subjects

	Tertiles of estimated glomerular filtration rate			
	First (n = 204) > 72 ml/min per 1.73 m²	Second (n = 205) 63 to 72 ml/min per 1.73 m²	Third (n = 204) < 63 ml/min per 1.73 m²	P value (trend)
Estimated glomerular filtration rate (ml/min per 1.73 m²)	80 ± 7	68 ± 3	55 ± 8	< 0.001
Estimated creatinine clearance (ml/min per 1.73 m²)	86 ± 16	74 ± 12	62 ± 14	< 0.001
Serum creatinine (µmol/L)	82 ± 11	90 ± 12	104 ± 24	< 0.001
Male gender (n [%])	133 [65]	109 [53]	135 [66]	0.84
Age (years)	62 ± 7	63 ± 7	67 ± 6	< 0.001
Normal glucose metabolism (n [%])	77 [38]	94 [46]	79 [39]	0.84
Impaired glucose metabolism (n [%])	56 [28]	60 [29]	57 [28]	0.91
Type 2 diabetes (n [%])	71 [35]	51 [25]	68 [33]	0.75
Body mass index (kg/m^2)	26.7 ± 3.6	26.8 ± 3.5	28.1 ± 4.4	< 0.001
Waist-to-hip ratio (cm/cm)	0.94 ± 0.09	0.90 ± 0.08	0.92 ± 0.08	< 0.01
Total cholesterol (mmol/L)	6.5 ± 1.2	6.7 ± 1.2	6.7 ± 1.2	0.06
High-density lipoprotein cholesterol (mmol/L)	1.3 ± 0.4	1.3 ± 0.3	1.3 ± 0.3	0.98
Lipid-lowering medication (n [%])	5 [2]	1 [0]	3 [1]	0.41
Systolic blood pressure (mmHg)	138 ± 20	136 ± 19	144 ± 19	< 0.01
Diastolic blood pressure (mmHg)	83 ± 10	82 ± 10	83 ± 10	0.82
Pulse pressure (mmHg)	55 ± 15	54 ± 13	62 ± 16	< 0.001
Blood pressure-lowering medication (n [%])	52 [25]	40 [20]	77 [40]	< 0.01
Hypertension (n [%])	108 [53]	90 [44]	136 [67]	< 0.01
Current smokers (n [%])	63 [31]	52 [25]	41 [20]	< 0.05
Prior cardiovascular disease (n [%])	47 [23]	43 [21]	52 [26]	0.56
Homocysteine (µmol/l)	11.2 ± 4.2	12.5 ± 5.9	13.9 ± 6.3	< 0.001
Von Willebrand factor (IU/ml)	110 (88 – 162)	108 (74 – 166)	134 (89 – 188)	< 0.05
Soluble vascular cell adhesion molecule-1 (µg/L)	1304 ± 423	1325 ± 368	1512 ± 515	< 0.001
Urinary albumin-creatinine ratio (mg/mmol)	0.77 (0.54 – 1.21)	0.87 (0.59 – 1.28)	0.99 (0.65 – 1.72)	< 0.05
C-reactive protein (mg/L)	1.75 (0.88 – 3.44)	1.49 (0.59 – 3.02)	2.13 (1.02 – 4.27)	0.74
Soluble intercellular adhesion molecule-1 (µg/L)	495 ± 190	462 ± 149	488 ± 146	0.67

Values for continuous variables are presented as mean ± SD or median (interquartile range). P value for trend was calculated with univariate analysis of variance for continuous variables and with χ^2 test for dichotomous variables. Hypertension was defined as a blood pressure ≥ 140 mmHg systolic and/or ≥ 90 mmHg diastolic and/or the current use of blood pressure-lowering medication.

Relationship between renal function and endothelial function

Table 2 shows that eGFR was independently associated with vWf, sVCAM-1, and ACR in all models (see also Figure 1, A through C).

Table 2. Relation of renal function with markers of endothelial function

	Model		Von Willebrand factor		Soluble vascular cell adhesion molecule-1		Urinary albumin-creatinine ratio	
			Stand. β (95% CI)		Stand. β (95% CI)		Stand. β (95% CI)	
1	Age, gender, glucose tolerance status	Estimated GFR	–0.11*	–0.20 to –0.02	–0.23‡	–0.32 to– 0.15	–0.20‡	–0.28 to –0.12
		Estimated CC	–0.02	–0.15 to 0.10	–0.15†	–0.24 to –0.05	–0.13†	–0.23 to –0.02
		Serum creatinine	0.07	0.00 to 0.14	0.25‡	0.17 to 0.34	0.27‡	0.19 to 0.35
2	Model 1 + prior CVD, systolic BP, current smoking, BMI, total cholesterol, Hcy	Estimated GFR	–0.10*	–0.19 to –0.001	–0.18‡	–0.27 to –0.10	–0.14†	–0.22 to –0.06
		Estimated CC	–0.05	–0.18 to 0.07	–0.16†	–0.28 to –0.05	–0.17†	–0.28 to –0.06
		Serum creatinine	0.05	0.00 to 0.15	0.18‡	0.09 to 0.27	0.17‡	0.09 to 0.25
3	Model 2 + C-reactive protein, soluble intercellular adhesion molecule-1	Estimated GFR	–0.09*	–0.18 to 0.002	–0.14†	–0.22 to –0.05	–0.15‡	–0.23 to –0.08
		Estimated CC	–0.05	–0.17 to 0.08	–0.11*	–0.22 to –0.01	–0.18†	–0.29 to –0.07
		Serum creatinine	0.04	–0.03 to 0.11	0.11*	0.02 to 0.19	0.18‡	0.10 to 0.25

Von Willebrand factor, urinary albumin-creatinine ratio and C-reactive protein were transformed to natural logarithm (ln). Stand., standardized; CI, confidence interval; GFR, glomerular filtration rate; CC, creatinine clearance; CVD, cardiovascular disease; BP, blood pressure; BMI, body mass index; Hcy, homocysteine. *P < 0.05, †P < 0.01, ‡P < 0.001.

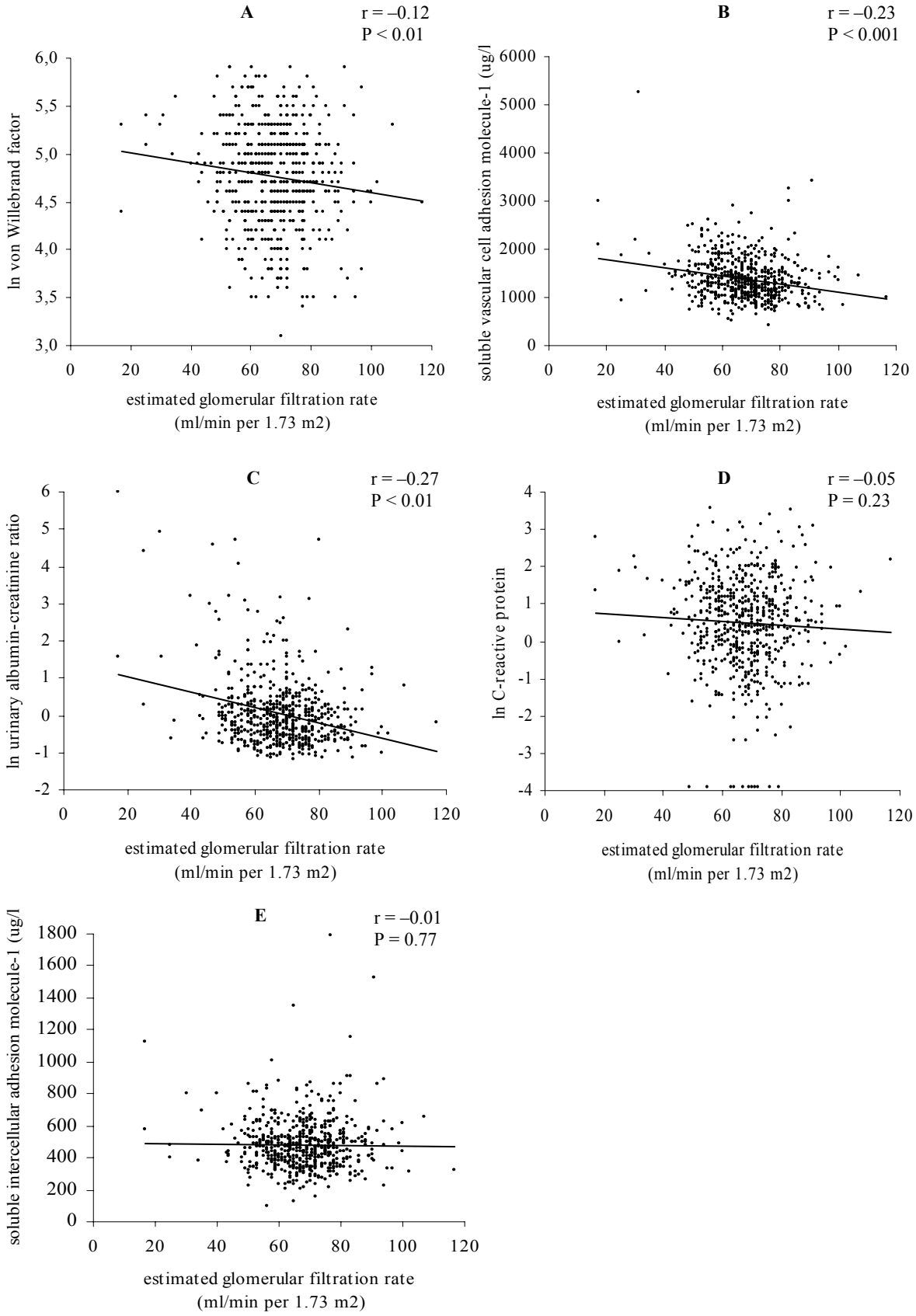

Figure 1. Relations between estimated glomerular filtration rate and markers of endothelial dysfunction (von Willebrand factor [A], soluble vascular cell adhesion molecule-1 [B], urinary albumin-creatinine ratio [C]) and inflammatory activity (C-reactive protein [D], soluble intercellular adhesion molecule-1 [E]). r, correlation coefficient.

Relationship between renal function and inflammatory activity

None of the inflammatory markers was related independently to any of the estimates of renal function (Table 3 and Figures 1, D and E). In model 1, a significant positive association was found between CRP and eCC, which, however, was not present after adjustment for BMI (standardized β 0.03; 95% CI -0.07 to 0.14; P = 0.58), one of the dependent variables in models 2 and 3. Body mass index was higher in participants with a higher eGFR (Table 1) and strongly positively associated with CRP (model 3: 0.20; 95% CI 0.11 to 0.28; P < 0.001).

Table 3. Relation of renal function with markers of inflammatory activity

Model			C-reactive protein		Soluble intercellular adhesion molecule-1	
			Stand. β (95% CI)		Stand. β (95% CI)	
1	Age, gender, glucose tolerance status	Estimated GFR	0.00	–0.07 to 0.08	–0.02	–0.10 to 0.07
		Estimated CC	0.14*	0.04 to 0.25	–0.03	–0.12 to 0.07
		Serum creatinine	0.03	–0.07 to 0.12	0.07	–0.01 to 0.16
2	Model 1 + prior cardiovascular disease, systolic blood pressure, current smoking, BMI, total cholesterol, homocysteine	Estimated GFR	0.04	–0.05 to 0.13	0.02	–0.08 to 0.11
		Estimated CC	0.04	–0.06 to 0.15	–0.03	–0.15 to 0.09
		Serum creatinine	–0.01	–0.09 to 0.08	0.01	–0.08 to 0.11
3	Model 2 + von Willebrand factor, soluble vascular cell adhesion molecule-1, urinary albumin-creatinine ratio	Estimated GFR	0.07	–0.03 to 0.16	0.06	–0.03 to 0.15
		Estimated CC	0.07	–0.06 to 0.20	0.02	–0.14 to 0.10
		Serum creatinine	–0.04	–0.12 to 0.05	–0.03	–0.12 to 0.06

C-reactive protein, von Willebrand factor and urinary albumin-creatinine ratio were transformed to natural logarithm (ln). Stand., standardized; CI, confidence interval; GFR, glomerular filtration rate; CC, creatinine clearance; BMI, body mass index. *P < 0.01.

Renal function, endothelial dysfunction and inflammatory activity in relation to cardiovascular and all-cause mortality

Median duration of follow-up was 12.5 yr (interquartile range 9.9 to 13.2). During follow-up, 192 of 613 individuals died (67 of cardiovascular disease and 31 of unknown causes). Renal function, whether expressed as eGFR, eCC, or serum creatinine, was inversely associated with risk of cardiovascular and all-cause mortality. After adjustment for age, gender, glucose tolerance, systolic blood pressure and previous cardiovascular disease, the RR (95% CI) of cardiovascular and all-cause mortality associated with a decrease of 5 ml/min per 1.73 m^2 of eGFR were 1.22 (1.09 to 1.36) and 1.12 (1.05 to 1.20), respectively (Tables 4 and 5). The relationship between eGFR and cardiovascular mortality is shown in Figure 2. The association was stronger for eGFR than for eCC or serum creatinine (Table 4).

When vWf, sVCAM-1, and ACR were added individually to the model, the RR for cardiovascular mortality associated with renal function decreased from 1.22 to 1.21, 1.16 and 1.17, respectively (Table 4, models 3 through 5). When the three markers were added simultaneously, the RR decreased from 1.22 to 1.12 (Table 4, model 6). Adjustment for vWf

and sVCAM-1 without ACR decreased the RR from 1.22 to 1.16 (Table 4, model 7). After addition of sICAM-1 to the three endothelial markers, the RR decreased from 1.22 to 1.13 (Table 4, model 8). The RR for all-cause mortality associated with renal function was not materially affected by these markers of endothelial dysfunction (Table 5). Adjustment for markers of inflammatory activity did not change substantially the RR for cardiovascular (Table 4) or all-cause (Table 5) mortality associated with renal function. Sequential inclusion of smoking status, BMI (or hip-to-waist ratio), total cholesterol (or high-density lipoprotein cholesterol), use of lipid-lowering medication, and homocysteine as independent variables in model 2 of the Cox regression analysis did not materially change the effect of adjustment for the different markers of endothelial function and/or inflammation on the renal function-associated cardiovascular and all-cause mortality (data not shown).

Table 4. Relative risks of cardiovascular mortality associated with estimates of renal function

Model		Estimated GFR	Estimated CC	Serum creatinine
		RR (95% CI) associated with a decrease of 5 ml/min per 1.73 m^2	RR (95% CI) associated with a decrease of 5 ml/min per 1.73 m^2	RR (95% CI) associated with a increase of 5 µmol/l
1	Age, gender, glucose tolerance status	1.22‡ (1.09 – 1.36)	1.10* (1.01 – 1.21)	1.11‡ (1.07 – 1.16)
2	Model 1 + prior CVD, systolic blood pressure	1.22‡ (1.09 – 1.36)	1.12* (1.02 – 1.23)	1.11‡ (1.06 – 1.16)
3	Model 2 + vWf	1.21† (1.08 – 1.35)	1.12* (1.02 – 1.23)	1.10‡ (1.06 – 1.15)
4	Model 2 + sVCAM-1	1.16† (1.04 –1.30)	1.08 (0.99 – 1.19)	1.09‡ (1.04 – 1.14)
5	Model 2 + ACR	1.17† (1.04 – 1.31)	1.09 (0.99 – 1.19)	1.09‡ (1.04 – 1.14)
6	Model 2 + vWf, sVCAM1, ACR	1.12* (1.00 – 1.26)	1.07 (0.98 – 1.17)	1.08† (1.02 – 1.13)
7	Model 6 – ACR	1.16* (1.04 – 1.30)	1.09 (0.99 – 1.19)	1.09‡ (1.04 – 1.14)
8	Model 6 + sICAM-1	1.13* (1.01 – 1.27)	1.06 (0.97 – 1.17)	1.07† (1.02 – 1.13)
9	Model 2 + CRP	1.22† (1.09 – 1.36)	1.12* (1.02 – 1.23)	1.11‡ (1.06 – 1.16)
10	Model 2 + sICAM-1	1.20† (1.08 – 1.34)	1.10 (1.00 – 1.21)	1.10‡ (1.05 – 1.15)
11	Model 2 + CRP, sICAM-1	1.20† (1.07 – 1.34)	1.10* (1.00 – 1.22)	1.10‡ (1.05 – 1.15)

Von Willebrand factor, urinary albumin-creatinine ratio and C-reactive protein were transformed to natural logarithm (ln). GFR, glomerular filtration rate; CC, creatinine clearance; RR, relative risk; CI, confidence interval; CVD, cardiovascular disease; vWf, von Willebrand factor; sVCAM1, soluble vascular cell adhesion molecule-1; ACR, urinary albumin-creatinine ratio; sICAM-1, soluble intercellular adhesion molecule-1; CRP, C-reactive protein. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

Table 5. Relative risks of all-cause mortality associated with estimates of renal function

Model		Estimated GFR	Estimated CC	Serum creatinine
		RR (95% CI) associated with a decrease of 5 ml/min per 1.73 m^2	RR (95% CI) associated with a decrease of 5 ml/min per 1.73 m^2	RR (95% CI) associated with a increase of 5 µmol/l
1	Age, sex, glucose tolerance status	1.12† (1.04 – 1.19)	1.03 (0.98 – 1.09)	1.08‡ (1.04 – 1.11)
2	Model 1 + prior CVD, systolic blood pressure	1.12† (1.05 – 1.20)	1.04 (0.98 – 1.09)	1.08‡ (1.04 – 1.11)
3	Model 2 + vWf	1.11† (1.04 – 1.19)	1.04 (0.99 – 1.10)	1.07‡ (1.04 – 1.11)
4	Model 2 + sVCAM-1	1.11† (1.04 – 1.19)	1.04 (0.98 – 1.10)	1.08‡ (1.04 – 1.11)
5	Model 2 + ACR	1.10† (1.03 – 1.18)	1.04 (0.99 – 1.10)	1.07‡ (1.04 – 1.11)
6	Model 2 + vWf, sVCAM1, ACR	1.10† (1.03 – 1.18)	1.05 (0.99 – 1.11)	1.07‡ (1.03 – 1.11)
7	Model 6 – ACR	1.10† (1.03 – 1.18)	1.04 (0.98 – 1.10)	1.07‡ (1.04 – 1.11)
8	Model 6 + sICAM-1	1.10† (1.03 – 1.18)	1.04 (0.98 – 1.10)	1.07‡ (1.03 – 1.10)
9	Model 2 + CRP	1.12† (1.05 – 1.20)	1.04 (0.98 – 1.10)	1.08‡ (1.04 – 1.11)
10	Model 2 + sICAM-1	1.11† (1.04 – 1.19)	1.03 (0.98 – 1.09)	1.07‡ (1.04 – 1.11)
11	Model 2 + CRP, sICAM-1	1.11† (1.04 – 1.19)	1.04 (0.98 – 1.10)	1.07‡ (1.04 – 1.11)

Von Willebrand factor, urinary albumin-creatinine ratio and C-reactive protein were transformed to natural logarithm (ln). GFR, glomerular filtration rate; CC, creatinine clearance; RR, relative risk; CI, confidence interval; CVD, cardiovascular disease; vWf, von Willebrand factor; sVCAM1, soluble vascular cell adhesion molecule-1; ACR, urinary albumin-creatinine ratio; sICAM-1, soluble intercellular adhesion molecule-1; CRP, C-reactive protein. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

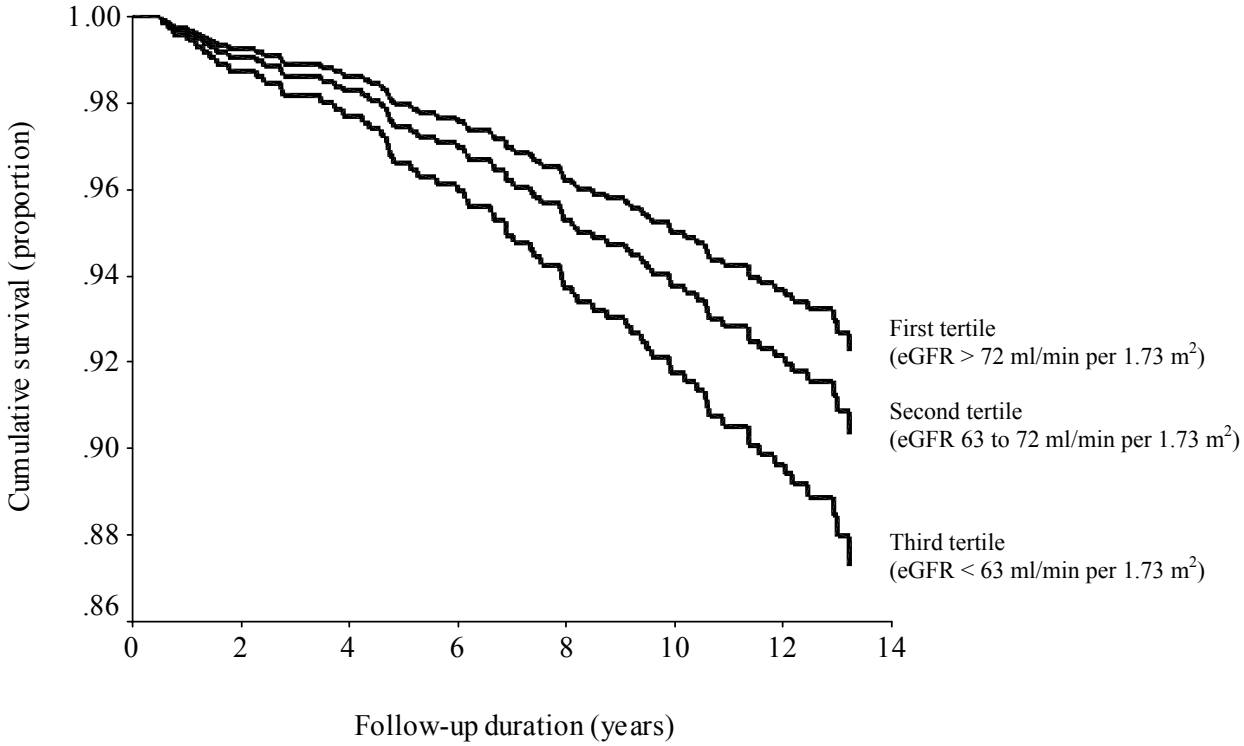

Figure 2. Proportion of subjects without cardiovascular death according to tertiles of estimated glomerular filtration rate, adjusted for age, sex and glucose tolerance status (Cox regression analysis). eGFR, estimated glomerular filtration rate.

Additional analyses

Because age and gender are part of the Cockcroft-Gault formula and the MDRD equation and were added to the analyses with serum creatinine as key independent variable, all models were tested without these (stratification) variables. This did not materially influence our results (data not shown).

In the multivariate and Cox regression analyses, replacement of systolic blood pressure by diastolic blood pressure, pulse pressure, presence of hypertension, or use of blood pressure-lowering medication did not materially change the relation between renal function and markers of endothelial dysfunction or inflammatory activity (data not shown). In addition, we repeated all analyses after exclusion of the four patients with chronic kidney disease stage 4 (eGFR 15 to 29 ml/min per 1.73 m^2) [1], which again did not materially change the results.

Further analyses to exclude the presence of effect modification by the presence of diabetes, older age (> 65 yr), male gender, or hypertension confirmed the consistency of the results, as the associations between renal function and biochemical markers of inflammatory activity and endothelial dysfunction and between eGFR and mortality were similar regardless of the presence or absence of these states (data not shown).

DISCUSSION

This population-based study had three main findings. First, mild impairment of renal function was independently associated with endothelial dysfunction. Second, we confirm and extend our previous finding [5] of an association between mild impairment of renal function and cardiovascular mortality (and to a lesser extent all-cause mortality) that was independent of conventional risk factors and that persisted during up to 13 yr of follow-up. Third, endothelial dysfunction seemed to be involved in this renal function-associated cardiovascular mortality.

An increasing number of studies show that individuals with even mildly impaired renal function are at high risk for cardiovascular morbidity and mortality, independent of traditional risk factors [2]. We have proposed that endothelial dysfunction and/or inflammation may be (partially) responsible for this phenomenon [14]. This was based on the general view that changes in endothelial cell properties and increased inflammation play an important role in the initiation and the progression of the atherothrombotic process [6] and on observations that patients with renal dysfunction show evidence of endothelial dysfunction and inflammation [13,14].

This study demonstrates that endothelial dysfunction is related to renal function in an elderly general population in whom renal function was only mildly impaired but cannot establish whether endothelial dysfunction causes impairment of GFR or vice versa or that a third variable causes both. Endothelial dysfunction may be an important mechanism linking mildly impaired renal function to cardiovascular disease, because healthy endothelium normally has antiatherothrombotic properties, such as promotion of vasodilatation and inhibition of vascular smooth muscle cell proliferation, thrombosis, and inflammatory activity. Many of these functions are mediated by the release of compounds with specific biological properties, such as nitric oxide, proteins involved in hemostasis and fibrinolysis, adhesion molecules, and selectins. The plasma concentrations of such substances are thought to reflect endothelial function status. Von Willebrand factor, which has prothrombogenic properties through its involvement in platelet adhesion and aggregation and in blood coagulation [23], has been suggested to be a marker of generalized endothelial dysfunction [24]. Vascular cell adhesion molecule-1 is involved in the recruitment of mononuclear blood cells into the vascular wall [25]. Soluble VCAM-1 in plasma is derived from shedding of endothelial cells, and has also been related to endothelial dysfunction [26]. In the absence of significant excretion of sVCAM-1 in the urine [27], plasma sVCAM-1 concentration can be

regarded as a reflection of endothelial release. Finally, microalbuminuria is often regarded as a marker of generalized endothelial dysfunction and has been shown to be independently associated with impaired endothelium-dependent vasodilatation [28]. In accordance with the concept that these markers reflect endothelial dysfunction, vWf, sVCAM-1, and (micro)albuminuria have all been associated with increased risk for cardiovascular and all-cause mortality [7–9].

This is the first population-based study to show that mildly impaired renal function is associated with endothelial dysfunction. Instead, some studies have suggested that microalbuminuria is associated with glomerular *hyper*filtration [29,30]. For example, in the PREVEND study, individuals with microalbuminuria, as compared with those with normoalbuminuria, had a higher mean creatinine clearance (97 versus 90 ml/min per 1.73 m^2) [29]. In contrast, we observed a *linear* inverse relationship between eGFR and ln ACR. This discrepancy may be related to the higher mean age and the lower mean eGFR in the current compared to these previous studies [29,30].

In our study, no significant relation was found between renal function and inflammation, as estimated with sICAM-1 and CRP. In patients with more advanced predialysis renal insufficiency, plasma sICAM-1 [14,31] and CRP [14,32] have been shown to be elevated. The relationship between mildly impaired renal function and CRP is less clear. In another community-based, non-diabetic population, Stuveling et al. [33] found that CRP was related with both diminished and high creatinine clearance (i.e., a U-shaped relationship). As in our study, the positive relationship between CRP and eCC disappeared after adjustment for BMI. This is probably due to the strong positive relationship between CRP and BMI [34,35], which may be explained by the finding that interleukin-6, a regulator of the synthesis of CRP in the liver, has been shown to be secreted by human subcutaneous adipose tissue [36]. As neither serum creatinine nor eGFR was significantly related with CRP, it is unlikely that a true association between CRP and eCC was masked by overadjustment for BMI.

Mild renal function impairment was associated with cardiovascular and all-cause mortality during up to 13 yr of follow-up, in a way that was independent of conventional risk factors for cardiovascular disease. Recent studies, with a shorter follow-up, have also demonstrated that mildly decreased GFR is an independent risk factor for cardiovascular disease [3,4]. Compared to our previous study of the same cohort [5], the present study consisted of older individuals, had a longer follow-up and more mortality cases, and, therefore, more power to identify and quantify influences of individual risk factors on cardiovascular and all-cause mortality. A major and new finding of our study is that the association of renal function and

cardiovascular mortality was attenuated after adjustment for endothelial function, suggesting that the relationship may be partially explained by endothelial dysfunction.

It is likely that the change in RR for cardiovascular mortality associated with a decrease in eGFR of 5 ml/min per 1.73 m^2 from 1.22 to 1.12, which was caused by adjustment for biochemical markers of endothelial dysfunction, is an underestimation of the true impact of endothelial dysfunction in individuals with mildly impaired renal function. The markers of endothelial dysfunction that we used reflect only selected aspects of a complex biological entity; for example, these markers contain no (or at most very indirect) information on endothelial nitric oxide synthesis, which is known to be impaired in individuals with impaired renal function [37] and which is likely to play an important role in the association between impaired renal function and atherothrombosis [38]. In this respect, it is noteworthy that the three endothelial function markers we used (vWf, sVCAM-1, and ACR) contributed *mutually independently* to the association between impaired renal function and cardiovascular mortality, suggesting that these various aspects of endothelial dysfunction all are relevant. Furthermore, misclassification of the 31 patients who had an unknown cause of death may have caused underestimation of the impact of endothelial dysfunction on cardiovascular mortality.

We used ACR as a marker of endothelial dysfunction. However, it has been suggested that microalbuminuria may sometimes reflect increased albumin excretion by the kidney without generalized endothelial dysfunction [21,39]. An alternative explanation of our data, therefore, is that decreased eGFR and increased ACR are related to cardiovascular mortality through similar mechanisms that (in part) have nothing to do with endothelial dysfunction. We cannot exclude this, but the RR of cardiovascular mortality decreased also when we adjusted for endothelial function assessed by vWf and sVCAM-1 without ACR, although to a lesser extent.

In this study, there was no relation between markers of inflammation and renal function or renal function-associated mortality. However, such relations may have been underestimated, because only CRP and sICAM-1 were used as parameters of inflammation. Although both are predictors of cardiovascular disease [8,10,11], they may only partially reflect the state of chronic inflammation. Whether other important markers of inflammation (*e.g.*, tumor necrosis factor-α, interleukin-6, secretory phospholipase A_2) explain renal insufficiency-associated cardiovascular mortality requires further investigation. A limitation of our data is that we studied a relatively healthy, elderly white population. Therefore, it remains to be investigated

whether the results of our study can be generalized to individuals who are younger, nonwhite, or at high risk for cardiovascular morbidity and mortality.

CONCLUSION

In this population-based study, endothelial dysfunction, but not inflammation was related to renal function and seemed to contribute to cardiovascular mortality in mild renal insufficiency. The results of this study may be clinically relevant because endothelial dysfunction is potentially reversible [40]. In the prevention of cardiovascular disease in mild renal insufficiency, targeting of endothelial dysfunction therefore might be an early treatment goal.

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Chapter 9

Summary and discussion

BACKGROUND OF THE THESIS

Patients with chronic kidney disease have an elevated risk of cardiovascular morbidity and mortality, which cannot be totally accounted for by traditional cardiovascular risk factors, such as hypertension, diabetes mellitus, dyslipidaemia and smoking. Therefore, alternative risk factors seem to be operative and deserve attention. In this thesis, we examined the role of homocysteine, endothelial dysfunction, low-grade inflammation and advanced glycation end-product-peptides as potential risk factors for cardiovascular disease in patients with chronic kidney disease.

SUMMARY

Homocysteine

Elevated plasma levels of homocysteine, a sulphur-containing amino acid, are independently associated with a higher risk of coronary heart disease and stroke in the general population. As plasma homocysteine levels are elevated in patients with renal dysfunction, it has been proposed that hyperhomocysteinaemia contributes to cardiovascular disease in end-stage renal disease patients. Some prospective studies have indeed found that hyperhomocysteinaemia is associated with negative cardiovascular outcomes in end-stage renal disease, but other studies have found no significant or even an opposite effect. The mechanism underlying the elevated plasma homocysteine level in renal failure is incompletely understood. In ***Chapter 2***, we argue that the hyperhomocysteinaemia encountered in end-stage renal disease is not likely to be explained by a fall in renal capacity to excrete or extract homocysteine, because in healthy individuals plasma homocysteine levels in the renal artery and vein are similar, and the urinary homocysteine excretion is negligible. Instead, we hypothesized that a uraemic disturbance in whole body methionine-homocysteine metabolism could account for the increase in plasma homocysteine in renal failure.

In sulphur amino acid metabolism, the essential amino acid methionine is converted to homocysteine in the transmethylation pathway, with S-adenosylmethionine and S-adenosylhomocysteine as intermediates (Chapter 1, Figure). Next, homocysteine can either receive a methyl group to regenerate methionine in the remethylation pathway, or be (irreversibly) degraded in the transsulphuration pathway. Flux rates of methionine

transmethylation, homocysteine remethylation and homocysteine transsulphuration may be influenced by plasma levels of B-vitamins, homocysteine metabolites (S-adenosylmethionine and S-adenosylhomocysteine) and genetic polymorphisms of enzymes involved in homocysteine metabolism. Homocysteine metabolism can be studied with stable-isotope techniques using infusions of [$^{2}H_3$-methyl-l-^{13}C]methionine as tracer, which enables calculation of whole body flux rates of methionine transmethylation, homocysteine remethylation and homocysteine transsulphuration in steady state and their determinants in end-stage renal disease patients. Additional calculation of the metabolic homocysteine clearance by transsulphuration (transsulphuration rate divided by plasma total homocysteine concentration) gives information about the whole body homocysteine disposal capacity, which is not adequately reflected by the absolute transsulphuration rate.

In ***Chapter 3***, we show that methionine-homocysteine metabolism in patients with end-stage renal disease is characterized by an impaired metabolic homocysteine clearance by transsulphuration and remethylation. In addition, the data suggest that the absolute rates of remethylation and transmethylation are diminished in end-stage renal disease patients compared to healthy individuals and are negatively related to whole blood S-adenosylhomocysteine levels in the whole group of patients with end-stage renal disease and healthy individuals. We hypothesize that the elevated intracellular concentration of S-adenosylhomocysteine in end-stage renal disease mediates the impairment in remethylation and transmethylation flux rates.

In ***Chapter 4***, we show that oral treatment with folic acid, a synthetic precursor of 5-methyltetrahydrofolate, (1) lowers, but does not normalize, plasma homocysteine concentration, (2) increases whole body homocysteine remethylation and methionine transmethylation rate to normal values, and (3) does not significantly affect transsulphuration rate or metabolic homocysteine clearance by transsulphuration in end-stage renal disease patients. The resistance to folic acid of hyperhomocysteinaemia in end-stage renal disease in the presence of normalized remethylation and transmethylation fluxes suggests a persistent defect in metabolic homocysteine clearance by transsulphuration as the cause of this hyperhomocysteinaemia.

In ***Chapter 5***, we show that, also in healthy subjects, treatment with folic acid increases (1) the rates of whole body homocysteine remethylation and methionine transmethylation, (2) decreases plasma total homocysteine concentration, and (3) does not significantly alter homocysteine transsulphuration or metabolic homocysteine clearance by transsulphuration. In addition, it was demonstrated that folic acid increased the plasma S-adenosylmethionine/S-

adenosylhomocysteine ratio, which from in vitro studies is known to stimulate the activity of the methyltransferases involved in methionine transmethylation. Remarkably, the decrease in plasma homocysteine concentration did not correlate with the increase in remethylation and/or transmethylation.

Endothelial dysfunction and inflammatory activity

The endothelium is not only a barrier to blood constituents, but is a biologically active interface that modulates tone, growth, haemostasis, and inflammation throughout the circulatory system. Endothelial dysfunction is seen in the early phases of atherosclerosis. Endothelial dysfunction is characterized by high plasma concentrations of endothelium-derived proteins and impaired endothelium-dependent vasodilatation. Several studies have demonstrated that endothelium-dependent vasodilatation is decreased in patients with advanced or end-stage renal disease. On the other hand, there are relatively few studies in patients with renal disease that have examined blood levels of endothelium-derived proteins, such as von Willebrand factor, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, tissue-type plasminogen activator, plasminogen activator inhibitor-1 and E-selectin. Furthermore, it is unknown whether the relationship between endothelial dysfunction and renal function is linear.

Evidence accumulates that atherosclerosis may be an inflammatory disease. An inflammatory response, produced by infection, has been observed to induce endothelial dysfunction in healthy humans. Inflammation is characterized by increased production of inflammatory molecules, named cytokines (e.g. tumour necrosis factor-α and interleukin-6), by white blood cells. These cytokines cause a change in the production of acute phase proteins by the liver which can rise (e.g. C-reactive protein and fibrinogen) or fall (e.g. albumin). In addition, cytokines induce the production of secretory phospholipase A_2 and stimulate the expression of soluble intercellular adhesion molecule-1 on endothelial cells. Thus, C-reactive protein, secretory phospholipase A_2 and soluble intercellular adhesion molecule-1 may be used as biochemical markers of the activity of different processes of the inflammatory response. There are only scarce data on the relationship between renal function and inflammatory status.

To explore the association between creatinine clearance and endothelial function and inflammatory activity (both reflected by multiple biochemical markers), mean SD (Z-)scores for markers of endothelial function and inflammatory activity can be calculated. This approach was developed to reduce the influences of biological variability of separate

measurements, and to reduce the number of associations to be investigated. For calculation of a Z-score, for every individual, each variable has to be expressed as the standard deviation of the difference from the population mean. The Z-score is calculated as the mean of these standard deviations.

In ***Chapter 6***, we show that impaired renal function is associated with endothelial dysfunction (as reflected by the endothelial function Z-score constructed from von Willebrand factor, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, type plasminogen activator, plasminogen activator inhibitor-1 and E-selectin) and inflammatory activity (as reflected by the inflammatory activity Z-score constructed from C-reactive protein and secretory phospholipase A_2) in 80 non-diabetic patients with a renal function ranging from normal to a pre-dialysis situation. These associations were linear and therefore present in patients with a renal function ranging from pre-dialysis to normal creatinine clearance.

In ***Chapter 8***, as a sequel of the patient-based study of Chapters 6, we studied individuals of the Hoorn Study population. In this population-based study composed of 613 individuals with a predominantly mildly impaired renal function (mean estimated glomerular filtration rate 68 ± 12 ml/min per 1.73 m^2), we showed that endothelial dysfunction (estimated by von Willebrand factor, soluble vascular cell adhesion molecule-1, and the urinary albumin-creatinine ratio), but not low-grade inflammation (estimated by plasma C-reactive protein and soluble intercellular adhesion molecule-1), was related to renal function. Further we showed that renal function was an independent predictor of cardiovascular and all-cause mortality. Endothelial dysfunction, but not inflammatory activity, contributed to the renal function-associated cardiovascular mortality.

Advanced glycation end-products

Advanced glycation end-products are a heterogeneous group of compounds derived from the non-enzymatic reaction between glucose or other reducing sugars and proteins. Resistance to complete enzymatic degradation of advanced glycation end-products leads to the formation of advanced glycation end-product-peptides, which can be measured more easily than advanced glycation end-products. Advanced glycation end-products may be involved in atherogenesis (1) by interaction with specific advanced glycation end-product receptors, leading to cytokine production, and (2) by their accumulation in the vascular matrix, possibly interfering with endothelial function. Accumulation of advanced glycation end-products and advanced glycation end-product-peptides in plasma has been described in end-stage renal

disease and diabetes mellitus. However, little is known about the relation between advanced glycation end-products or advanced glycation end-product-peptides on the one hand and endothelial dysfunction and inflammatory activity on the other hand in pre-dialysis renal insufficiency.

In ***Chapter 7***, we show that plasma concentrations of advanced glycation end-product-peptides are independently related to creatinine clearance over a wide range of renal function in the same patient group as in Chapter 6. Advanced glycation end-product-peptides were not independently related to biochemical markers of endothelial dysfunction (von Willebrand factor, soluble vascular cell adhesion molecule-1, soluble E-selectin, tissue-type plasminogen activator and plasminogen activator inhibitor-1, and their compound Z-score) and inflammatory activity (soluble intercellular adhesion molecule-1, C-reactive protein, secretory phospholipase A_2, and their compound Z-score). Therefore, the supposed atherogenic effect of advanced glycation end-products seems not to be mediated by mechanisms reflected by these markers.

DISCUSSION

The studies presented in this thesis have limitations which are discussed below.

In ***Chapter 2***, considering the putative mechanisms of hyperhomocysteinaemia in chronic kidney disease, one has to be aware of the many uraemia-related factors that can influence homocysteine metabolism (e.g. protein malnutrition, vitamin deficiency, older age, drugs), of which probably many have not yet been identified.

In ***Chapter 3***, comparing whole body homocysteine metabolism of end-stage renal disease patients with healthy individuals proved to be difficult, because the small number of individuals and the relative mismatch in age, hampered robust conclusions. In addition, the stable-isotope infusion technique is an elegant but costly and time-consuming method to quantify flux rates in homocysteine metabolism, which in itself has some limitations. First, because of the prerequisite of a steady state during stable-isotope measurements, individuals were studied during rest and in fasting condition. Therefore, generalization to the situation in real life, in which humans are ambulatory and postprandial most of the time is difficult. Second, in the stable-isotope model, samples of extracellular compartments are used to reflect processes that (partly) take place in the cell. Therefore, the use of samples of breath air and blood for calculation of whole body homocysteine metabolism, may cause underestimation of

the contribution of intracellular homocysteine metabolism. To minimize this possible effect, we corrected the flux rates for a bicarbonate retention factor and checked by multiple samples taking that a plateau in stable-isotope enrichment was reached (and therefore an equilibrium between intra- and extracellular concentrations). Another limitations is that the stable-isotope study of the whole body homocysteine metabolism does not provide organ specific information. Therefore, disturbances of the homocysteine metabolism in an organ are underestimated when evaluating whole body metabolism.

In ***Chapter 4 and 5***, studying the effect of folic acid intervention on whole body homocysteine metabolism in end-stage renal disease patients and healthy individuals is limited by the prerequisite of a steady state in the stable-isotope model. Thus, changes in flux rates and concentrations or tissue distributions of metabolites might have been transient and therefore not have been picked up. By example, a possible homocysteine lowering mechanism of folic acid, which cannot be detected by the stable-isotope model, could be an increase in homocysteine remethylation, which initially would not be accompanied by an increase in methionine transmethylation. The resulting rise in (re-)generated methionine could be stored in the cell or be used for protein synthesis. Finally, methionine transmethylation would increase until it is in balance with homocysteine remethylation, and homocysteine plasma level finds a new steady state, but, at a lower level as before folic acid treatment. The increased transmethylation rate induced by folic acid may be clinically relevant despite the fact that plasma homocysteine concentration remains elevated in end-stage renal disease patients. It has been shown that folic acid treatment decreases DNA hypomethylation in end-stage renal disease patients without normalizing plasma homocysteine concentration and evidence is mounting that DNA hypomethylation may play an important part in the development of cardiovascular disease. The non-significant relation between plasma homocysteine and transmethylation, which seems to be present in healthy individuals as well, suggests that intervention trials with homocysteine-lowering treatment aimed at cardiovascular risk reduction should be cautious to use only plasma homocysteine as intermediary biochemical end-point. In addition, the relationship between the folic acid dose and changes in whole body homocysteine flux rates are unknown and deserve further study.

In ***Chapter 6***, the study of the relation between endothelial dysfunction and inflammatory activity on the one hand and creatinine clearance on the other hand is hampered by the fact that there is no gold standard of endothelial dysfunction and inflammatory activity. We used a selection of biochemical markers of endothelial dysfunction which are generally accepted to reflect endothelial dysfunction, but we did not measure endothelium-dependent

vasodilatation. The markers of inflammatory activity we used are thought to reflect only a small part of the inflammatory process (acute phase response by C-reactive protein, leucocyte adhesion to the endothelium by soluble intercellular adhesion molecule-1, and synthesis of eicosanoids by secretory phopholipase A_2). Measurement of cytokines (e.g. tumour necrosis factor-α and interleukin-6) that are initially produced in the inflammatory process could give more sensitive but perhaps less specific information about the presence of inflammation. Another possible limitation of this study was that the intra-individual variability of the markers of endothelial dysfunction and inflammatory activity was not assessed. However, the use of a composite Z-score of these markers should have lessened a masking effect (if any) on the associations that were studied. Further, the with the Cockcroft-Gault formula calculated creatinine clearance is a rather crude estimate of the glomerular filtration rate, even after correction of the body surface area. In addition, the heterogeneity of the underlying renal diseases restricts interpretations of the results and the findings cannot be extrapolated to the general population. Finally, the cross-sectional character of the study does not allow conclusions regarding causality of the relations that were found.

In ***Chapter 7***, in the same study population as in Chapter 6, the relation of advanced glycation end-product-peptides with creatinine clearance and biochemical markers of endothelial dysfunction and inflammatory activity was studied. Next to the same limitations as in Chapter 6, the use of advanced glycation end-product-peptides as reflection of advanced glycation end-products is arguable. However, the online spectrophotometric and spectrofluorometric method to detect advanced glycation end-product-peptides that was used in this study, has been validated with an advanced glycation end-product-Elisa, which showed a strong correlation between advanced glycation end-product-peptides and the major advanced glycation end-products N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxyethyl)lysine.

In ***Chapter 8***, the fact that, in contrast to the patient-based study of Chapter 6, no significant relationship could be demonstrated between biochemical markers of inflammatory activity and renal function in this population-based study was remarkable. A possible explanation could be found in the finding that, at approximately the same creatinine clearance, the patients in Chapter 6 had higher blood levels of the markers of inflammatory activity (C-reactive protein and soluble intercellular adhesion molecule-1) than the individuals from the general population in Chapter 8. This suggests (1) that an unknown patient-related factor might cause a state of (clinically unrecognized) inflammation and (2) that a higher grade of inflammation is necessary to cause renal insufficiency or, again, renal function impairment may cause more inflammation in susceptible individuals, e.g. patients. Another explanation

could be that the biochemical marker of inflammatory activity that correlated most strongly with renal function in the patients of Chapter 6 (secretory phopholipase A_2) was not measured in the population-based Hoorn Study described in Chapter 8.

CONCLUSIONS AND IMPLICATIONS FOR THE FUTURE

We showed that the hyperhomocysteinaemia encountered in end-stage renal disease is probably caused by a disturbance in the whole body homocysteine metabolism that is characterized by an impaired homocysteine remethylation, methionine transmethylation and metabolic homocysteine clearance by transsulphuration, which is accompanied by an increase in S-adenosylhomocysteine and (to a lesser extent) S-adenosylmethionine. The culprit in the homocysteine metabolism in end-stage renal disease cannot be identified with experiments performed in steady state, but a defect in the transsulphuration pathway would be a plausible candidate and could explain our main findings. A primary decrease in homocysteine transsulphuration would cause accumulation of plasma homocysteine and its precursors (S-adenosylhomocysteine more than S-adenosylmethionine) that on the one hand could stimulate homocysteine transsulphuration until it again equilibrates methionine intake (but at a higher homocysteine level) and on the other hand could inhibit methionine transmethylation and homocysteine remethylation. The finding that folic acid intervention was able to normalize homocysteine remethylation and methionine transmethylation, with a decrease (but not normalization) of plasma homocysteine and an unaffected metabolic homocysteine clearance by transsulphuration is consistent with this hypothesis.

Further we showed that endothelial dysfunction, inflammation and advanced glycation end-product-peptides are related to renal function in patients with pre-dialysis chronic kidney disease. In a population-based study with a predominantly mildly impaired renal function, we showed that endothelial dysfunction, but not low-grade inflammation, is related to renal function and contributed to the renal function-associated cardiovascular mortality.

The relation between renal function and cardiovascular disease is complex. This may be due to renal function-associated confounders, that can affect the relation between cardiovascular risk factors and cardiovascular disease. By example, in chronic kidney disease, so far, prospective studies did not unequivocally demonstrate the predictive value for cardiovascular disease of high blood levels of homocysteine and advanced glycation end-products. This does not per se reflect an altered homocysteine-mediated pathophysiology for

cardiovascular disease in chronic kidney disease, but more likely, reflects a confounding factor like malnutrition, which on the one hand has been associated with renal function impairment and on the other hand with low blood levels of homocysteine and advanced glycation end-products. Studies that investigate the relation between a cardiovascular risk factor or treatment in end-stage renal disease should, where possible, correct for the many potential confounders associated with uraemia, especially protein energy malnutrition.

The exact mechanism through which hyperhomocysteinaemia may lead to atherosclerosis is still unclear. It has been suggested that hypomethylation of DNA or other substances may play a role in the development of vascular disease. In fact, it has been shown that folic acid in end-stage renal disease not only lowers homocysteine, but also decreases DNA hypomethylation. When DNA hypomethylation, probably caused by the decreased transmethylation rate in end-stage renal disease, is the pathophysiological phenomenon leading to cardiovascular disease, hyperhomocysteinaemia would be an important indicator of this atherogenic process. We found that folic acid-induced changes in plasma homocysteine were not significantly related to changes in whole body transmethylation rates in healthy individuals. This suggests that plasma homocysteine may not be the parameter of choice for measuring the effect of treatments that aim for reduction of DNA hypomethylation. The latter may better be evaluated by recently developed methods that can measure the methylation status of DNA in e.g. leucocytes.

A problem in the studies investigating endothelial dysfunction and inflammation is that there is no gold standard. For example, endothelial dysfunction may not only be reflected by biochemical markers in plasma (from which we used a selection in the studies of this thesis), but also by endothelium-dependent vasodilatation. Furthermore, some markers not only reflect endothelial dysfunction, but also other pathophysiological processes. For example, albuminuria not only reflects endothelial dysfunction but also changes in renal anatomy and haemodynamics. Soluble intercellular adhesion molecule-1 might reflect inflammation as well as endothelial dysfunction. We propose the combined use of multiple markers for the analyses of endothelial dysfunction as well as inflammation. Analysis of individual markers will give specifically information about a limited aspect of endothelial dysfunction and inflammation, whereas combined analysis of markers with mean standard deviation (Z-)scores will give more sensitive information whether and to which extent endothelial dysfunction and/or inflammation are present.

The finding that the relation between inflammation and renal function was present in patients but could not be demonstrated in a larger group of individuals of a larger population

underscores the carefulness with which conclusions from a study population may be generalized to other populations, and the importance of the selection of the marker(s) of inflammatory activity.

Future studies that aim to explore the relation between renal function and cardiovascular disease should measure a broad range of markers of endothelial dysfunction and inflammatory activity, correct for traditional cardiovascular risk factors as well as for potential confounders associated with uraemia, like malnutrition, and include new intermediary endpoints, like DNA methylation.

Samenvatting

voor degenen die niet met het onderwerp bekend zijn

ACHTERGROND VAN HET PROEFSCHRIFT

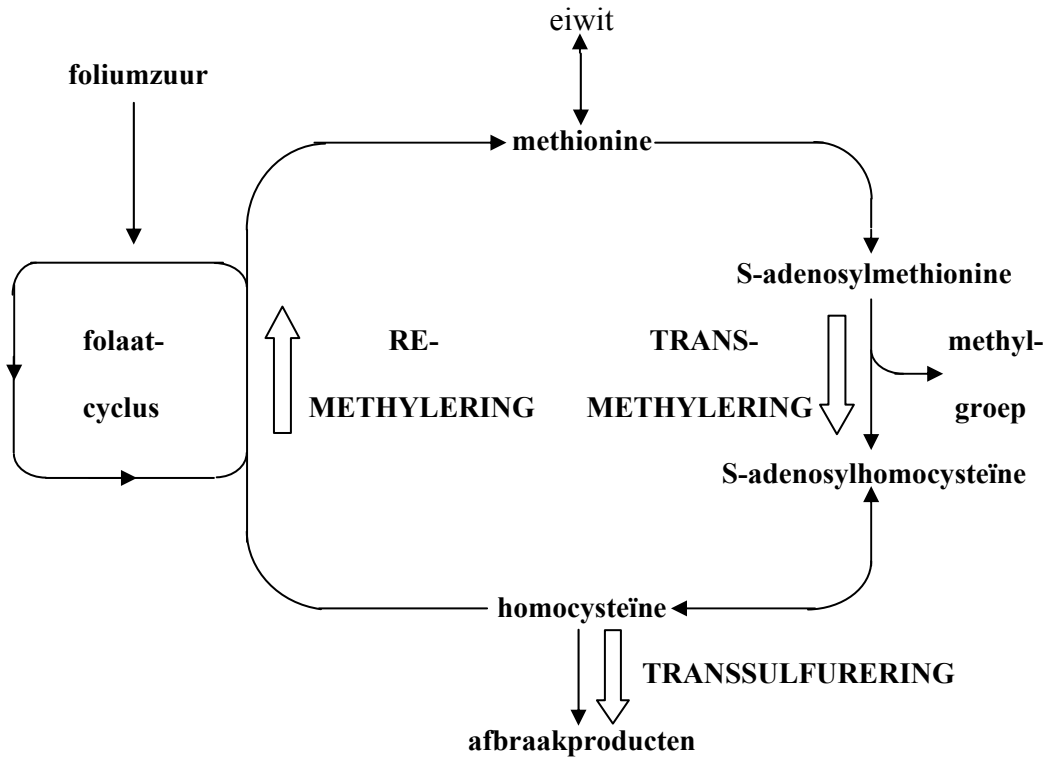
Patiënten met chronische nierziekten hebben een verhoogd risico op hart- en vaatziekten. Algemeen bekende risicofactoren voor hart- en vaatziekten, zoals hoge bloeddruk, diabetes mellitus, verhoogd cholesterolgehalte en roken, lijken de overmaat aan hart- en vaatziekten bij mensen met chronische nierziekten niet volledig te kunnen verklaren. De onderzoeken in dit proefschrift hebben zich gericht op homocysteïne, vaatwanddisfunctie, chronische ontsteking en 'advanced glycation end-product'-peptiden als mogelijke risicofactoren voor met nierfunctieverlies samenhangende hart- en vaatziekten.

SAMENVATTING

Homocysteïne

Homocysteïne is een aminozuur. De meeste aminozuren zijn bouwstenen van eiwitten, maar homocysteïne niet. Homocysteïne ontstaat wanneer een ander aminozuur, methionine, gebruikt wordt om koolstofatomen toe te voegen aan stoffen in het lichaam. Hyperhomocysteïnemie (verhoogde concentratie van homocysteïne in het bloed) voorspelt het optreden van hart- en vaatziekten in de algemene bevolking. Omdat hyperhomocysteïnemie samenhangt met een gestoorde nierfunctie, wordt verondersteld dat hyperhomocysteïnemie bijdraagt aan de ontwikkeling van hart- en vaatziekten bij mensen met een chronische nierziekte. De ontstaanswijze van hyperhomocysteïnemie bij chronische nierziekten is echter onopgehelderd. In ***Hoofdstuk 2*** wordt beargumenteerd dat het onwaarschijnlijk is dat hyperhomocysteïnemie bij chronische nierziekten wordt veroorzaakt door het verlies van het vermogen van de nieren om homocysteïne via de urine uit te scheiden of in de nier af te breken. In plaats daarvan wordt gesteld dat een gestoorde homocysteïnestofwisseling de oorzaak zou kunnen zijn van hyperhomocysteïnemie bij chronische nierziekten.

In de homocysteïnestofwisseling (Figuur 1), ontstaat homocysteïne door afsplitsing van een koolstofbevattende methylgroep van het essentiële aminozuur methionine (transmethylering), waarbij S-adenosylmethionine en S-adenosylhomocysteïne tussenproducten zijn. Homocysteïne kan vervolgens door toevoeging van een door folaat of betaïne gedoneerde methylgroep worden omgezet in methionine (remethylering) óf onomkeerbaar worden afgebroken (transsulfurering). Door gebruik te maken van niet-radioactief gemarkeerd methionine, dat via een infuus in het lichaam wordt gebracht, kunnen de snelheden van de



Figuur. Vereenvoudigde weergave van de homocysteïnestofwisseling. De open pijlen geven de in dit proefschrift gemeten omzettingsprocessen binnen de homocysteïnestofwisseling aan.

methioninetransmethylering, homocysteïneremethylering en homocysteïnetranssulfurering worden berekend. Het vermogen van het lichaam om zich te ontdoen van homocysteïne (verder homocysteïneklaring genoemd) wordt weergegeven door de verhouding van de homocysteïnetranssulfureringssnelheid en de homocysteïneconcentratie in het bloed.

Hoofdstuk 3 toont dat patiënten met terminaal nierfalen (eindstadium van een nierziekte, waarbij nierfunctievervangende therapie noodzakelijk is geworden) een veel lagere homocysteïneklaring door transsulfurering hebben dan gezonde vrijwilligers. Daarnaast blijken de snelheden van de methioninetransmethylering en homocysteïneremethylering verlaagd te zijn. Er wordt gesteld dat de bij terminaal nierfalen verhoogde S-adensylhomocysteïneconcentratie waarschijnlijk de oorzaak is van de gestoorde methioninetransmethylering en homocysteïneremethylering.

Hoofdstuk 4 laat zien dat behandeling met foliumzuur (dat in het lichaam wordt omgezet in folaat) bij patiënten met terminaal nierfalen de hyperhomocysteïnemie gedeeltelijk corrigeert, de methioninetransmethylering en homocysteïneremethylering normaliseert, maar de homocysteïnetranssulfurering en de homocysteïneklaring door transsulfurering niet verandert. Dit impliceert een foliumzuuronafhankelijk defect in de homocysteïnetranssulfurering als oorzaak van de resterende hyperhomocysteïnemie.

Hoofdstuk 5 demonstreert dat behandeling met foliumzuur bij gezonde vrijwilligers de homocysteïneremethylering en methioninetransmethylering stimuleert en de homocysteïne-concentratie verlaagt, maar de homocysteïnetranssulfurering en de homocysteïneklaring door transsulfurering niet significant verandert. Tevens neemt de verhouding S-adenosylmethionine/S-adenosylhomocysteïne toe, waarvan door andere onderzoekers in (buiten het lichaam uitgevoerde) experimenten is aangetoond dat dit de methioninetransmethylering stimuleert. Opvallend is het ontbreken van een aantoonbaar verband tussen de mate van daling van de homocysteïneconcentratie en de toename van de remethylerings- en transmethyleringssnelheid.

Vaatwanddisfunctie en chronische ontsteking

De vaatwand is niet slechts een barrière voor het bloed, maar heeft een belangrijke biologische functie bij de regulatie van de vaatwandspanning, vaatgroei en ontstekingsactiviteit in het gehele vaatstelsel. Vaatwanddisfunctie wordt gezien in de vroege fase van hart- en vaatziekten en wordt gekenmerkt door verhoogde concentraties in het bloed van bepaalde in de vaatwand geproduceerde eiwitten én door een gestoorde vaatverwijding na stimulatie van de vaatwand. Verschillende studies hebben deze gestoorde vaatwandafhankelijke vaatverwijding aangetoond bij terminaal nierfalen. Er is echter slechts in beperkte mate onderzoek verricht naar de relatie tussen chronische nierziekte en van de vaatwand afkomstige eiwitten in het bloed.

Steeds meer onderzoeken ondersteunen de stelling dat hart- en vaatziekten ontstekingsprocessen zijn. Er is aangetoond dat een door een infectie uitgelokte ontstekingsreactie kan leiden tot vaatwanddisfunctie. Bij een ontsteking worden cytokines (ontstekingsmoleculen) geproduceerd door witte bloedcellen. Deze cytokines veranderen de productiesnelheid van zogenaamde acute-fase-eiwitten in de lever. Daarnaast stimuleren cytokines de productie en expressie van sommige eiwitten in de vaatwandcel. De concentratie in het bloed van acute-fase-eiwitten en door de vaatwandcel geproduceerde eiwitten weerspiegelt dus de mate van ontsteking in het lichaam.

Om de relatie tussen enerzijds nierfunctie (in dit proefschrift vereenvoudigd gedefinieerd als de mate waarin de nieren afvalstoffen uitscheiden) en anderzijds vaatwanddisfunctie en ontsteking te onderzoeken (beiden weerspiegeld door bovengenoemde eiwitten), werd in dit proefschrift gebruik gemaakt van een scoresysteem. Met dit scoresysteem kunnen de concentraties van de verschillende eiwitten, die respectievelijk de vaatwanddisfunctie en de

ontstekingactiviteit weerspiegelen, worden samengevat in een score, waardoor met één getal de mate van vaatwanddisfunctie of ontstekingactiviteit kan worden weergegeven.

In ***Hoofdstuk 6*** wordt bij 80 niet-diabetische patiënten (met een normale tot ernstige nierfunctiestoornis) aangetoond dat een verminderde nierfunctie samenhangt met zowel vaatwanddisfunctie als ontsteking. Dit verband is aanwezig in het gehele nierfunctiebereik.

In ***Hoofdstuk 8*** wordt, als vervolg op hoofdstuk 6, bij 613 mensen uit een bevolkingsonderzoek in Hoorn (met een overwegend gering gestoorde nierfunctie) aangetoond dat vaatwanddisfunctie, maar niet ontsteking, gerelateerd is aan de nierfunctie. Een gestoorde nierfunctie blijkt een voorspeller te zijn van sterfte door hart- en vaatziekten, waarbij vaatwanddisfunctie, in tegenstelling tot ontstekingsactiviteit, bijdraagt aan de met de nierfunctie samenhangende oversterfte ten gevolge van hart- en vaatziekten.

'Advanced glycation end-products'

'Advanced glycation end-products' (AGEs) zijn stoffen die ontstaan bij niet-enzymatische reacties tussen suikers en eiwitten. Een onvolledige afbraak van AGEs resulteert in vorming van AGE-peptiden (eenvoudige verbindingen van aminozuren), die in het bloed gemakkelijker te bepalen zijn dan AGEs. AGEs zouden door het op gang brengen van een ontstekingreactie en door AGE-ophoping in de vaatwand tot vaatwanddisfunctie en mogelijk uiteindelijk ook hart- en vaatziekten kunnen leiden. Bij mensen met terminaal nierfalen en diabetes mellitus is stapeling van AGEs en AGE-peptiden in het bloed aangetoond. Bij niet-diabetische mensen met een minder ernstige nierfunctiestoornis is echter weinig bekend over de relatie tussen AGEs en AGE-peptiden enerzijds en vaatwanddisfunctie en ontsteking anderzijds.

Hoofdstuk 7 laat zien dat de bloedconcentratie van AGE-peptiden gerelateerd is aan de nierfunctie bij de patiënten uit hoofdstuk 6. De bloedconcentratie van AGE-peptiden is echter niet gerelateerd aan vaatwanddisfunctie en ontsteking. Het lijkt daarom dat het veronderstelde schadelijke effect van AGEs niet ontstaat via vaatwanddisfunctie en ontsteking.

CONCLUSIES EN BETEKENIS VOOR DE TOEKOMST

Concluderend lijkt hyperhomocysteïnemie bij terminaal nierfalen te worden veroorzaakt door een verstoring van de homocysteïnestofwisseling. Dit wordt gekenmerkt door een verminderde homocysteïneremethylering, methioninetransmethylering en homocysteïne-

klaring door transsulfurering, die gepaard gaat met een toename van S-adenosylhomocysteïne en S-adenosylmethionine. De oorspronkelijke afwijking in de homocysteïne-stofwisseling bij chronische nierziekten kan met ons onderzoek niet met zekerheid worden aangetoond, maar een primair gestoorde homocysteïnetranssulfurering zou op theoretische gronden echter veel, zo niet alle, bovengeschetste afwijkingen kunnen verklaren. Het feit dat de behandeling met foliumzuur resulteerde in normalisatie van de homocysteïneremethylering en methioninetransmethylering, bij een nog steeds (zij het in mindere mate) verhoogde homocysteïneconcentratie en verlaagde homocysteïneklaring door transsulfurering, ondersteunt deze hypothese.

Daarnaast laat dit proefschrift zien dat homocysteïne, vaatwanddisfunctie, ontsteking en AGE-peptiden gerelateerd zijn aan de nierfunctie bij patiënten met een normale tot ernstig gestoorde nierfunctie. Bij mensen uit de algemene bevolking met een merendeels milde nierfunctiestoornis is vaatwanddisfunctie eveneens gerelateerd aan de nierfunctie, maar ontsteking niet. In deze laatste groep blijkt vaatwanddisfunctie bij te dragen aan de met de nierfunctie samenhangende oversterfte ten gevolge van hart- en vaatziekten.

De relatie tussen nierfunctie en hart- en vaatziekten is complex. Dit wordt mede veroorzaakt door het gegeven dat sommige met een chronische nierziekte samenhangende situaties niet alleen een relatie vertonen met risicofactoren voor hart- en vaatziekten, maar ook met hart- en vaatziekten zelf. Zo heeft men, in tegenstelling tot bij gezonde vrijwilligers, bij mensen met een chronische nierziekte nog niet aan kunnen tonen dat een hoge bloedconcentratie van homocysteïne en AGEs het optreden van hart- en vaatziekten voorspelt. Dit betekent niet per se dat het ontstaan van hart- en vaatziekten bij mensen met een chronische nierziekte anders verloopt dan bij mensen met een normale nierfunctie, maar lijkt eerder te wijzen op een verstorende variabele, zoals ondervoeding, die zowel samenhangt met de aanwezigheid van een chronische nierziekte als een lage bloedconcentratie van homocysteïne en AGEs. Studies die de relatie tussen nierfunctie en hart- en vaatziekten onderzoeken zouden, indien mogelijk, rekening moeten houden met dergelijke verstorende variabelen.

Het exacte mechanisme waarlangs hyperhomocysteïnemie tot hart- en vaatziekten leidt is nog niet opgehelderd. Mogelijk speelt een gestoorde capaciteit van het lichaam om via methioninetransmethylering methylgroepen af te staan, hetgeen nodig is voor herstel van beschadigd eiwit en DNA, een rol. Inmiddels is door anderen aangetoond dat foliumzuurtoediening bij mensen met terminaal nierfalen niet alleen de hyperhomocysteïnemie doet afnemen, maar ook de DNA-methylering verbetert. Bovendien

zijn er aanwijzingen dat een lage DNA-methyleringsgraad de ontwikkeling van hart- en vaatziekten kan bevorderen.

Een probleem bij studies die vaatwanddisfunctie en ontsteking onderzoeken is dat er voor beide processen geen “gouden standaard” bestaat. Zo kan bijvoorbeeld voor meting van vaatwanddisfunctie, naast de in dit proefschrift gebruikte bloedconcentratie van vaatwandafgeleide eiwitten, ook echografisch de vaatwandafhankelijke vaatverwijding worden gemeten. Daarnaast geven sommige vaatwandafgeleide eiwitten niet alleen informatie over de vaatwandfunctie, maar ook over andere processen in het lichaam (waaronder ontsteking!). Het verdient daarom de voorkeur bij de bestudering van vaatwanddisfunctie en ontsteking gebruik te maken van een combinatie van verschillende meetinstrumenten.

De bevinding dat het verband tussen ontsteking en nierfunctie wel aantoonbaar was bij patiënten en niet bij gezonde vrijwilligers onderstreept het belang van voorzichtigheid bij generalisatie van conclusies die gebaseerd zijn op onderzoek van een specifieke populatie.

Toekomstige studies die zich richten op de relatie tussen nierfunctie en hart- en vaatziekten zouden vaatwanddisfunctie en ontsteking met meerdere methoden moeten meten. Verder zou rekening moeten worden gehouden met bekende risicofactoren voor hart- en vaatziekten en mogelijke verstorende variabelen die samenhangen met een gestoorde nierfunctie. Tot slot verdient het aanbeveling nieuwe eindpunten en pathofysiologische paden te onderzoeken, zoals bijvoorbeeld de DNA-methyleringsgraad.

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Vader en moeder. Jullie hebben mij de vrijheid en mogelijkheid gegeven om geneeskunde te gaan studeren. Zonder jullie geen proefschrift.

Eleonoor, Deborah en Ymke. Liefsten, iedere keer wanneer ik thuis kom weet ik weer waar ik het voor doe!

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De auteur van dit boekje werd geboren op 13 januari 1966 te Heemskerk. Van 1978 tot 1984 bezocht hij het Bonhoeffer College te Castricum, waar hij het VWO-diploma behaalde. Het doctoraalexamen geneeskunde en het artsexamen werden respectievelijk in 1988 en 1991 cum laude afgelegd aan de Vrije Universiteit te Amsterdam. De opleiding tot internist werd in zijn geheel in het Vrije Universiteit medisch centrum te Amsterdam gevolgd en in 1998 afgerond (opleider: Prof.dr. J. van der Meer). In 1998 werd hij in de Westfälische Wilhelms-Universität te Münster (Duitsland) opgeleid tot echografist (opleider: Prof.dr. K.H. Rahn). Sindsdien werkt hij als internist in het Vrije Universiteit medisch centrum (afdelingshoofd: Prof.dr. S.A. Danner) en werd hij in 2002 geregistreerd in het aandachtsgebied vasculaire geneeskunde.